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Interaction of naturally occurring and synthetic MSH peptides with peripheral and CNS melanocortin receptors

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INTERACTION OF NATURALLY OCCURRING AND SYNTHETIC MSH PEPTIDES WITH PERIPHERAL AND CNS MELANOCORTIN RECEPTORS

submitted by Ulrike Gisela Sahm
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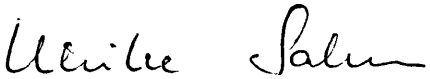

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Abbreviations

ACTH	adrenocorticotropic hormone
BSA	bovine serum albumin
CD	circular dichroism
DIC	diisopropylcarbodiimide
DNA	desoxyribonucleic acid
DOPA	3,4-dihydroxyphenylalanine
DTPA	diethylamine pentaacetic acid
EDT	1,2-ethane dithiol
EDTA	ethylendiaminetetraacetic acid
FAB-MS	fast atom bombardment mass spectroscopy
GABA	γ-amino butyric acid
HEPES	*N*-(2-hydroxyethyl)piperazine-N-2'-ethane sulphonic acid
HOBT	1-hydroxybenzotriazole
LDL	low density lipoprotein
LPH	lipotropin
MALDI-TOF MS	matrix assisted laser desorption ionisation-time of flight mass spectroscopy
MC receptor	melanocortin receptor
MEM	minimum essential medium
MeOH	methanol
MSH	melanocyte-stimulating hormone
NMR	nuclear magnetic resonance
Org 2766	$[Met(O_2)^4, D\text{-}Lys^8, Phe^9]ACTH_{(4\text{-}9)}$
PBS	phosphate buffered saline
POMC	pro-opiomelanocortin
TFA	trifluoroacetic acid

Amino acids

Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Cys	cysteine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
Leu	leucine
Lys	lysine
Met	methionine
Nle	norleucine
Phe	phenylalanine
Pro	proline
Ser	serine
Trp	tryptophan
Tyr	tyrosine
Val	valine

ABSTRACT

The work presented in this thesis is concerned with the way in which a small peptide hormone, α-MSH, interacts with specific receptors on the surface of target cells. A systematic investigation into the structure-activity relationships of the interaction of the hormone with the MC1 melanocortin receptor expressed by B16 murine melanoma cells has been undertaken. Suitable analogues of α-MSH were synthesised by solid-phase techniques and were assayed for receptor binding, stimulation of tyrosinase and induction of melanin biosynthesis. Different series of peptides tested included α-MSH fragments, alanine substituted derivatives, cyclic peptides and analogues containing D-amino acids.

The amino acids responsible for the interaction with the receptor were identified as Met^4, His^6, Phe^7, Arg^8 and Trp^9, and to a lesser extent, Pro^{12}. The charged side-chains of the Glu^5 and Lys^{11} did not seem to participate in either receptor binding or stimulation of biological activity.

Single amino acid replacement by alanine was recognised as the most valuable tool in investigating the role of individual residues. Therefore, the series of alanine-substituted α-MSH analogues was also tested for binding to a second melanocortin receptor, the MC3 receptor isolated from rat hypothalamus.

Binding to this brain receptor was determined by the same key residues (Met^4, His^6, Phe^7, Arg^8 and Trp^9) as affinity and activity on the MC1 receptor. An extra requirement exists for Tyr^2. As all these residues can be found in all MSH/ACTH peptides, it is therefore suggested that this receptor might recognise the common core sequence of all MSH peptides, $\alpha\text{-MSH}_{(4\text{-}9)}$.

Binding to both receptors was compared and a mechanism for the molecular interaction with the receptor is proposed.

CHAPTER 1

Introduction

The melanotropic peptides were among the first peptide hormones to be recognised and isolated, and have been extensively studied throughout this century. These studies, however, have almost exclusively been carried out in amphibia and reptiles, and their role in mammals has remained unclear until recently. A variety of new effects of MSH peptides has been discovered over the past fifteen years. The synthesis of potent and radiolabelled synthetic analogues made it possible to study the binding of the hormones to specific receptors and measure the biological response. The recent availability of the amino acid sequences of five subtypes of melanocortin receptors will allow insight into the molecular interaction of the hormone with the receptors. MSH peptides actively participate in skin pigmentation, and as receptors for melanotropic peptides are found in mammalian melanoma cells, they are of therapeutic and diagnostic interest in the treatment of the disease.

As malignant melanoma is resistant to most conventional therapies, it is mainly treated surgically but, because of its tendency to metastasise at an early stage, complete removal of the tumours is not always possible. It is therefore important to find a more efficient way of treating the disease as well as improving imaging techniques, both of which could be achieved potentially by a specific carrier that can target both drugs and imaging agents to melanoma cells. This could be a potential role for melanotropin analogues.

The rationale behind the concept of drug targeting is to achieve a desired pharmacological response at a selected site without undesired actions at non-target sites, or premature metabolism of the active drug (Gardner, 1985). To this aim, drug molecules have been linked to numerous classes of carriers, for example lectins, glycoproteins, enzymes, antibodies, poly- and oligo-peptides (see section *1.1.*) and DNA (see Gregoriadis, 1979; Goldberg, 1983), to achieve a more site-specific delivery of the molecule.

1.1. Peptides hormones as carriers in drug targeting

Small peptide hormones have been considered an alternative to antibodies and Fab fragments in drug targeting; their uptake into cells is receptor-mediated, and therefore specific for a certain type of cell. Also, peptide hormones do not possess the same immunogenic potential as antibodies being naturally occurring molecules, and of low molecular weight. However, the interactions of the hormone with other cell types expressing the target receptor or a receptor for a structurally related hormone have to be considered (e.g. receptors for ACTH in the case of α-MSH). If the drug-hormone conjugate is to be administered systemically, it has to be ensured that the drug concentration at the target site is high enough, and that the conjugate is not broken down in the plasma to give the free hormone and the free drug. There are certain pharmacokinetic problems associated with the use of peptides as drugs or carrier molecules, such as their poor bioavailability after oral administration, their poor uptake across biological membranes, and their rapid clearance.

An ideal peptide-drug conjugate would be rapidly and specifically taken up by the target cell; this requires a high affinity of the conjugate for the receptor, and a high rate of endocytosis/internalisation. Ideally, the receptor would be rapidly recycled and would participate in multiple rounds of delivery (Basu, 1990). Once

internalised, the conjugate would have to be broken down into the carrier and the active drug; with internalised ligand-receptor complexes, the site of this process would be the lysosome, and it has to be ensured that the active drug molecule then reaches the target compartment of the cell without being degraded.

A number of peptide hormones including α-MSH and β-MSH (see section *1.3.4*), have already been employed in drug targeting experiments; however, most of them have shown poor results when carried out *in vivo* (Chang, *et al.*, 1977; Oeltmann and Heath, 1979; Cowley *et al.*, 1980; Varga and Asato, 1977; Lejeune and Ghanem, 1993). One of the problems is that most of the receptors that have been well characterised (i.e. insulin-receptor, LDL receptors etc.) are present on a large number of different cell types, and selective delivery is therefore not possible.

1.2. Importance of understanding the structure-activity relationships

To make the best use of peptide carriers or targeting agents it is important to fully understand the way the molecule interacts with its receptor and the biological response it triggers. Knowledge of the organisation of the information within the molecule and, where possible, using this knowledge to optimise the molecule, are the key factors in the successful design and employment of peptide hormone drugs and carriers. The three-dimensional structures of a natural or synthetic small linear peptide are generally unpredictable, because they are too flexible to allow conformational studies by the conventional means of NMR and CD studies, likewise their biological properties are also unpredictable.

For example most of the biological activity of ACTH is retained by the 1-24 amino acid fragment; the remaining 15 amino acids appear to be dispensable (Schwyzer,

1977). This has been observed with a number of other peptide hormones and growth factors as well. Also in ACTH, it was observed, that within the 1-24 fragment, the first 10 amino acids appeared to be coding for the biological activity of the hormone, whereas the maximal binding affinity seemed to be determined by the 11-24 part of the molecule. This observation was the foundation of the so-called message-address concept (Schwyzer, 1977), which has since been successfully employed to design agonists and antagonists of naturally occurring peptides (Portoghese *et al.*, 1990). The influence of individual amino acids and their side-chains is usually investigated by single amino acid replacements with natural or synthetic amino acids and their analogues. The physico-chemical properties of the substituted amino acid are of great importance, and only one parameter (i.e. hydrophobicity, steric bulk, electronegativity etc.) should be changed at the time to allow an estimate of which properties are required in each position. Attempts have been made to quantify these influences, but, although this is a good model in theory, it is seldom possible to achieve (Fauchère, 1986).

Insight into the active conformation of the peptide can be obtained using spectroscopic methods, and also by testing conformationally restricted analogues for activity *in vitro*. Conformationally restricted analogues include both cyclic or cross-linked derivatives, and those where amino acids have been replaced by their D-isomer. It is agreed that the perfect complementarity between receptor and ligand is only achieved when the peptide is bound to the protein, however, it has been postulated that a preferred, energetically sensible conformation exists which is close to the one recognised by the receptor. Of course it is also possible that a number of low energy conformations exist, and the molecule only acquires the active conformation in the process of binding to the receptor.

1.3. Melanotropic Peptides

The melanotropic peptides are a group of structurally related pituitary peptide hormones that derive from the same precursor molecule, pro-opiomelanocortin (POMC). After excision of the signal peptide, POMC matures into β-lipotropin (β-LPH), ACTH and an N-terminal glycopeptide, which contains the γ-MSH sequence (Mains *et al.*, 1977; Crine *et al.*, 1978; Chrétien *et al.*, 1979; Crine *et al.*, 1979; Nakanishi *et al.*, 1979; Crine *et al.*, 1980; Gianoulakis *et al.*, 1980; Gossard *et al.*, 1980; Mains and Eipper, 1980; Miller *et al.*, 1980; Seidah *et al.*, 1981). ACTH is further processed to α-MSH and corticotropin-like intermediate-lobe peptide (CLIP), equivalent to $ACTH_{(18-39)}$. β-lipotropin is broken down into γ-lipotropin and β-endorphin, and those peptides are then processed to β-MSH and Met-Enkephalin, respectively (Fig. *1.1.*).

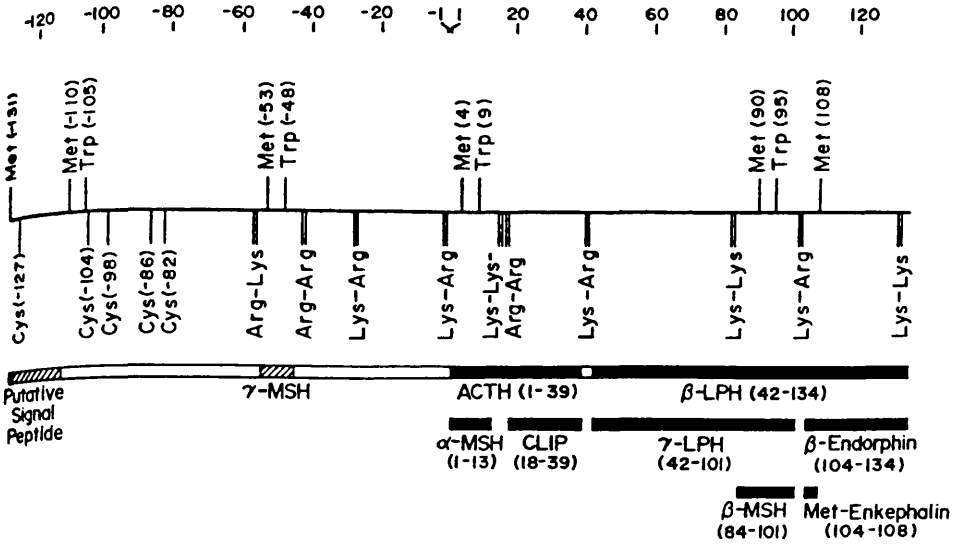


Fig. 1.1. Schematic representation of the bovine ACTH/β-LPH precursor as described by Nakanishi *et al.*, 1979.

MSH peptides, and α-MSH in particular, have been studied extensively over the past 80 years, and a number of extensive reviews are available (Eberle, 1988; Hadley *et al.*, 1988). Information contained in these volumes is therefore only mentioned briefly, and only when relevant for the discussion. Functions and biological properties of β-MSH and ACTH are not discussed in detail as these molecules are not part of this study.

1.3.1. α-MSH

Mammalian α-MSH (Ac-Ser^{1}-Tyr^{2}-Ser^{3}-Met^{4}-Glu^{5}-His^{6}-Phe^{7}-Arg^{8}-Trp^{9}-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH_2) is a basic tridecapeptide with an isoelectric point of 10.5-11.0. It also exists in its des-acetyl- or di-acetyl forms. Its amino acid sequence was first published in 1957 (Harris and Lerner). It is synthesised mainly in the *pars intermedia* of the pituitary gland, but there is evidence that some peripheral tissues are also able to synthesise the hormone. Biosynthesis and secretion of α-MSH in the pituitary are controlled by a variety of different inhibiting and excitatory factors. A number of hormones and neurotransmitters are involved in the control of α-MSH release, including dopamine, serotonin, GABA, somatostatin, noradrenaline and vasopressin (see Eberle, 1988, Chapter 10).

1.3.1.1. Distribution

In most species, α-MSH is mainly formed in the *pars intermedia* of the pituitary gland, or in the *pars distalis* in species that lack the *pars intermedia*. Within the *pars intermedia*, α-MSH- and β-MSH immunoreactivity is found within the same cells, whereas there was α-MSH detected in the corticotrophic cells in the *pars distalis*. In MSH producing cells, the peptides are detected together with non-POMC peptides such as gastrin, δ-sleep-inducing peptide and calcitonin-like immunoreactivity.

Rudman *et al.* studied the occurrence of MSH peptides in various regions of the rat, bovine and human brain (Rudman *et al.*, 1973, 1974). The highest concentration of the peptides was found in the hypothalamus, the lowest in the midbrain and the hippocampus. Several areas did not show the presence of MSH peptides.

α-MSH-like immunoreactivity can be detected in a variety of peripheral mammalian tissues, such as skin (Thody *et al.*, 1983), testes (Margionis *et al.*, 1983), placenta (Clark *et al.*, 1978) and the gastrointestinal tract (Fox and Kraicer, 1981). There is evidence that α-MSH in the skin and in the gastrointestinal tract might not be of hypophysial origin as the α-MSH content in hypophysectomized rats in these tissues does not differ from normal animals (Thody *et al.*, 1983; Fox and Kraicer, 1981). α-MSH can also be found in a number of malignant tumour cells (Morano and Estivariz, 1987; Hirata *et al.*, 1976) and elevated plasma levels of the hormone have been reported in some melanoma patients (Ghanem *et al.*, 1986).

1.3.1.2. Functions

Although the role of α-MSH has been more extensively studied in reptiles than in mammals, this chapter is mainly concerned with the way α-MSH interacts with pigment and melanoma cells. The mechanism of interaction with the MSH receptor is briefly described, and relevant functions in other tissues are mentioned. A good summary of the data up to 1988 is given by Eberle (1988), therefore it is not extensively reviewed in this chapter.

1.3.1.2.1. Mammalian melanocytes and melanoma cells

Mammalian melanocytes are derived from the neural crest, from where they migrate into the dermis as undifferentiated melanoblasts. Following invasion of the

dermis, they differentiate into melanocytes (Prota, 1967). Molecular mechanisms of the formation of melanins as well as their exact chemical structure are only partly understood. Eumelanin and phaeomelanin seem to be under different genetic control, but part of their biosynthetic pathway is the same (Fig.*1.2.*). Both originate from the same precursor, L-tyrosine, which in the first step is oxidised to L-DOPA and DOPAquinone. For the formation of phaeomelanin, this is then conjugated with glutathione or cysteine to cysteinyldopa, which is then oxidised to cysteinyldopaquinone, forms 1,4-benzothiazines and is processed in several steps to phaeomelanin. For eumelanin biosynthesis, the dopaquinone is processed to dopachrome, 5,6-dihydroxyindole and indole-5,6-quinone, which is able to form dimers called melanochrome. All these four molecules are involved in the formation of eumelanin (Prota, 1980). Tyrosinase (monophenol dihydroxy-phenylalanine:oxygen oxidoreductase; E.C. 1.14.18.1.) catalyses the first two steps of this pathway and is generally regarded as the key enzyme in melanogenesis. The expression of tyrosinase, however, is not variable in different skin types (Prota, 1992); therefore another regulating factor must be involved in melanin biosynthesis. Recent results suggest that (6R) 5,6,7,8-tetrahydrobiopterin might be participating in regulating the tyrosine supply to melanocytes as it is a cofactor for phenylalanine hydroxylase, the enzyme that catalyses the conversion of L-phenylalanine to L-tyrosine (Schallreuter *et al.*, 1994).

In cultured murine melanoma cells, internalisation of the receptor-ligand complex can be observed almost immediately after the binding to the receptor. It is believed that, once in the cell, the complex is degraded in the lysosome, as this procedure can be prevented by the acidotrophic agent NH_4Cl (Adams, 1993). Internalisation might be followed by down-regulation of the α-MSH receptors on the cell surface (Eberle, 1993). A much slower internalisation rate can be found in human melanoma cells (Siegrist *et al.*, 1993). An increase in adenylate cyclase activity is stimulated via a G protein within 5-20 min of the binding of the hormone to the

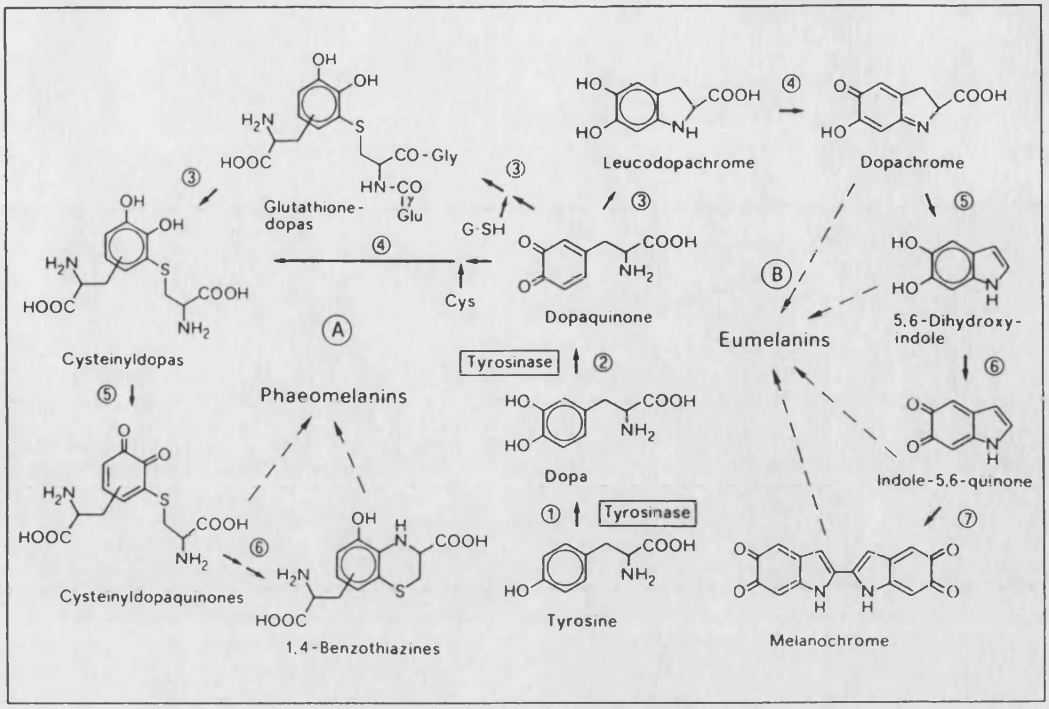


Fig. 1.2. Metabolic pathway from L-tyrosine to melanin. From: Eberle, 1988, p.231

receptor, reaching an early peak and then decreasing, before maximum binding is reached (Fuller *et al.*, 1979; Fuller *et al.*, 1987; deGraan *et al.*, 1987). From there, two possibly equally important signalling pathways are stimulated:, the activation of protein kinases and the stimulation of protein phosphorylation. The latter leads to hydrolysis of phosphatidyl-inositol-4,5-diphosphate, which ultimately leads to diacetylglycerol production and stimulation of protein kinase C (Buffey *et al.*, 1992); membrane bound protein kinase C appears rapidly with a peak response after 20-60 min, and then decreases. After 24h of exposure, levels of protein kinase C are back to residual activity. A phosphorylated 34kDa protein can be isolated from the culture medium after 5-20 min, whereas the time-course of the appearance of active tyrosinase is dependent on transcription.

There has been some interest in the relationship between melanogenic response and metastastic potential of the cells. Lunec *et al.* (1992) report that both melanogenesis and metastatic activity of the cells are increased by α-MSH peptides, which is surprising as metastatic activity is usually associated with loss of differentiation. Also, it is likely that both responses are mediated by the same receptor as melanoma cells seem to possess only one population of α-MSH receptors. It has been observed that the melanogenic response and metastatic activity might depend on different signalling mechanisms following activation of the receptor. The melanogenesis of B16 melanoma cells seemed to depend on the cell density, but the metastatic potential did not (Buffey *et al.*, 1992). Also, the Ca^{2+}-channel blocking agent verapamil enhances the melanogenesis without affecting the metastatic activity of B16 murine melanoma cells (Parker and Sherbet, 1993). This is in agreement with the finding that Ca^{2+} plays an important role in the receptor activation and cell signalling of melanocortin receptors in melanoma cells.

Ca^{2+} is believed to be equally important for receptor binding and signal transduction (Gerst and Salomon, 1987a,b). This effect can also be observed with ACTH receptors from adrenocortical cells (Cheitlin *et al.*, 1985) and might be a characteristic of this class of receptors. Recently, calmodulin-binding peptides have been shown to specifically inhibit the action of α-MSH on M2R melanoma cells (Eshel and Salomon, 1994).

In melanoma cells, α-MSH also affects the cell differentiation, proliferation and metastatic potential. It is not quite clear whether amelanotic melanoma cell lines form a better model regarding metastatic forms of melanoma. There have been reports of B16 murine melanoma cell subclones that lack the MSH receptor completely (Solca *et al.*, 1993). Most of these studies were carried out using

murine melanoma cells, but more recently, human melanoma cell lines have also been extensively studied.

α-MSH was reported to decrease the growth of Cloudman S91 melanoma cells whereas ACTH did not affect cell proliferation (Cobb and McGrath, 1974). However, in cells that are resistant to α-MSH, i.e. amelanotic lines, enhancement of the cell growth was observed (Halaban and Lerner, 1977). Similar observations were made when depleting normal S91 cells of tyrosine. With B16 murine melanoma, the same effects could be found (Niles and Logue, 1979) and are believed to be concentration dependent (Siegrist and Eberle, 1986).

1.3.1.2.2. Role of α-MSH in the central nervous system

The first report of MSH functions within the mammalian brain dates back to 1958, when Ferrari showed that intracisternal injection of MSH/ACTH peptides induced changes in the behaviour of dogs, rats, cats and rabbits (Ferrari, 1958). DeWied *et al.* showed that MSH/ACTH peptides improve avoidance learning in hypophysectomised rats (deWied *et al.*, 1964, 1965, 1966). Also MSH peptides were found to have an influence on the grooming behaviour of rats. This effect was shown to be dependent on the nature of the peptide: of all naturally occurring ligands, α-MSH had the strongest effect, followed by β-MSH and $ACTH_{(1-24)}$, whereas γ_2-MSH, $ACTH_{(4-10)}$ and [Leu^9]α-MSH were inactive. The synthetic, "superpotent" [Nle^4,D-Phe^7]α-MSH was even more active than α-MSH (Aloyo *et al.*, 1983).

α-MSH also shows some actions as a neurotransmitter, and it interferes with the cholinergic, (nor-)adrenergic, dopaminergic, serotoninergic and GABAergic systems (see Eberle, 1988).

Intravenous injection of either α-MSH or Org 2766, a synthetic ACTH analogue ([Met(O_2)^4, D-Lys^8, Phe^9]ACTH$_{(4-9)}$), has been reported to reduce the local permeability of the blood-brain barrier (Goldman and Murphy, 1981). The analogue Org 2766 also appears to facilitate nerve recovery from lesions and cisplatin-induced neurotoxicity (deKoning *et al.*, 1988).

Both MSH and ACTH were found to exhibit antipyretic action in pyrogen-induced fever and, given in higher dose, ACTH$_{(1-24)}$ and α-MSH also lowered the core temperature of afebrile rabbits (Glyn and Lipton, 1981; Murphy *et al.*, 1983). Centrally administered α-MSH was 25,000 times more active than acetaminophen (paracetamol); a mechanism which might be mediated by a functional antagonism between interleukin-1 and α-MSH. The tripeptide Ac-α-MSH$_{(11-13)}$ was also able to exhibit antipyretic effects, but it is less active than the full-length peptide (Richards *et al.*, 1984). D-amino acid replacement in positions 13 alone or 11 and 13 increased the antipyretic and antiinflammatory actions of the tripeptide, but replacement of proline in position 12 by its D-isomer left the peptide inactive (Hiltz *et al.*, 1991). In a study in rats, however, α-MSH and [Nle^4,D-Phe^7]α-MSH were shown to increase the core temperature rather than having a hypothermic effect (Raible and Knickerbocker, 1993).

α-MSH was shown to specifically inhibit the ability of interleukin-1 to participate in the proliferation of murine thymocytes and interleukin-1 induced synthesis of prostaglandin E in a dose-dependent way, whereas [Nle^4,D-Phe^7]α-MSH could not exhibit these effects (Cannon *et al.*, 1986). Centrally administered α-MSH could also antagonise the actions of the pro-inflammatory agents interleukin-1β, interleukin-8, leukotriene B_4 and platelet-activating factor (Ceriani *et al.*, 1994). In this study, α-MSH$_{(11-13)}$ was also active in inhibiting interleukin-1β.

Finally, α-MSH was found to modulate cardiac contractability, excitability and sensitivity to noradrenalin (Strand *et al.*, 1986), an effect which might be controlled by the CNS. A recent study suggested that the effects of $ACTH_{(1-24)}$ and [Nle^4,D-Phe^7]α-MSH on blood-pressure and the incidence of grooming behaviour might be causally related (Versteeg *et al.*, 1993).

The existence of these diverse functions of MSH in the brain indicate functionally distinct melanocortin receptors might exist. Two G protein-coupled melanocortin receptors found in the brain have recently been cloned and named MC3 and MC4 (Gantz *et al.*, 1993a,b; Roselli-Rehfuss *et al.*, 1993). There is further evidence of at least one more melanocortin receptor from the brain (Chhajlani *et al.*, 1993). Heterogeneity of the brain melanocortin receptors was also suggested by Tatro and Entwistle (1994), who performed *in situ* binding studies on tissues from different parts of the brain. α-MSH, desacetyl-α-MSH, β-MSH and ACTH were able to displace binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH from the binding sites, but the biologically active derivatives Org 2766, $ACTH_{(4-9)}$ and $\alpha\text{-MSH}_{(11-13)}$ could not inhibit the binding of the radiotracer.

1.3.1.3. Structure-activity relationships

Structure-activity relationships of α-MSH have been widely investigated, and are extensively reviewed in the literature. This section is meant to present a few main features of the structural interaction with pigment cells, but not a complete review of all the data available in the literature. Most of the studies concerning the structure-activity relationships of α-MSH have been carried out prior to 1988, and have been reviewed by Eberle (1988). More details on relevant information from previous studies are given in the appropriate discussion chapter (Chapter 4) and they are therefore not referred to individually here.

1.3.1.3.1. Terminal amino acids

Des-acetylation is reported to decrease the potency of the peptide approximately 10-fold in the frog skin assay, but not in other assay systems. Omission of the C-terminal amide does not alter the biological activity of the peptide. There has been some evidence that the C-terminal tripeptide might contain a "secondary message sequence" (Eberle and Schwyzer, 1975), but other studies showed that the tripeptide does not significantly increase the activity of some α-MSH fragments (Hruby *et al.*, 1987). N-terminal amino acids 1-3 are thought to be unimportant for the peptide's action, and Hruby's group frequently employ $\alpha\text{-MSH}_{(4\text{-}11)}$ analogues in their studies.

1.3.1.3.2. Core sequence

Within the "core sequence", the importance of the central heptapeptide $\alpha\text{-MSH}_{(4\text{-}10)}$ was recognised early on, but no systematic approach has been taken to elucidate the importance of individual residues. The glycine in position 10, however, was later shown to be replaceable (Medzihradsky, 1976) and acting as a spacer between the core sequence of the hormone and the C-terminal tripeptide. Thus, in this thesis, the core sequence is being referred to as $\alpha\text{-MSH}_{(4\text{-}9)}$. A number of amino acid replacements within this core sequence have been carried out with varying results. Substitution of Arg^8 led to significant loss of activity in all cases, whereas Met^4, His^6, Phe^7 and Trp^9 could be replaced by related amino acids and/or their isomers.

1.3.1.3.3. Cyclic peptides

Cyclic, conformationally restricted peptides are a valuable tool in studying the active conformation of a peptide. Several attempts have been made to cyclise α-MSH, and all the attempts made are discussed in detail in Chapter 4. Briefly, there are three main groups of cyclic peptides. The first derives from $[\overline{Cys^4,Cys^{10}}]\alpha\text{-MSH}$, a cyclic analogue that was first described in 1982 (Sawyer *et al.*, 1982).

This analogue exhibits slightly higher activity than α-MSH in the frog skin assay, and similar activities in all other assays. A number of fragments and analogues of [Cys^4,Cys^{10}]α-MSH have also been tested. Two other groups of cyclic peptides have been formed by introducing a lactam bridge into the molecule, and they derive from the general structures [Nle^4,D-Orn^5,Glu^8]α-MSH$_{(4-11)}$ (Sugg *et al.*, 1988) and [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{(4-10)}$ (Hadley *et al.*, 1989), respectively.

1.3.1.3.4. [Nle^4,D-Phe^7]α-MSH

Heat-alkali treatment of pituitary extracts has long been observed to prolong their melanotropic activity on melanophores (Lee and Buettner-Janusch, 1963). Sawyer *et al.* proposed that this might be due to racemisation, and as Met^4 and Phe^7 are more prone to racemisation than the other residues, they synthesised [Nle^4]α-MSH and [Nle^4,D-Phe^7]α-MSH for biological testing. If [Nle^4]α-MSH, where the residue in position 4 is no longer prone to racemisation, is exposed to hot alkali, the prolonged effect could nevertheless be observed. Thus, it had to be mediated by racemisation of the Phe^7 residue. [Nle^4,D-Phe^7]α-MSH proved to be more active than α-MSH, and showed prolonged effects in some of the assays (Sawyer *et al.*, 1980). It also shows a higher binding affinity, which is suggested to be due to "irreversible" binding to receptor, and a higher stability towards degradation and oxidation. [Nle^4,D-Phe^7]α-MSH can easily be radioiodinated at the tyrosine in position 2 without destroying the biological activity of the molecule.

[D-Phe^7]α-MSH has not been extensively studied, but seems to be almost equipotent to [Nle^4,D-Phe^7]α-MSH. It could therefore prove more suitable than [Nle^4,D-Phe^7]α-MSH for binding studies in human melanoma cells because of the higher non-specific binding associated with the Nle^4 residue suggested by Siegrist *et al.* (1993).

1.3.1.3.5. Antagonists

Desacetyl-α-MSH (McCormack *et al.*, 1982), α-$MSH_{(1-8)}$ (Eberle *et al.*, 1984) and several modified C-terminal peptides have been shown to partially inhibit the action of α-MSH at concentrations where they do not exhibit activity themselves. At a sufficiently high concentration they do however exhibit agonist activity. This has also been reported for γ-MSH (McCormack *et al.*, 1982). Ac-Nle-Asp-Trp-D-Phe-Nle-Trp-Lys-NH_2 has been described as a selective antagonist of α-MSH in the frog skin assay, but is inactive in the lizard skin assay (Al-Obeidi *et al.*, 1990). Cyclisation of this peptide between Asp and Lys restored its melanotropic activity. There are no antagonists described that inhibit α-MSH action in mammalian melanoma cells.

1.3.1.4. Use of α-MSH in tumour imaging and drug targeting

Several attempts have been made to employ MSH peptides as carrier molecules in selective drug delivery. The earliest study was by Varga *et al.* (1977), who conjugated β-MSH with daunomycin and achieved a three times higher toxicity compared to free daunomycin, while the conjugate was practically non-toxic to 3T3 fibroblasts. Later investigations focused more on the α-MSH molecule, presumably because it is shorter - and therefore cheaper to synthesise- and also exhibits a higher affinity for the melanoma cell MSH receptor.

Melanoma selective cytotoxicity was observed with a diphtheria toxin-related α-MSH fusion protein (Murphy *et al.*, 1986). This protein was produced by genetic engineering in *E. coli* and was tested against human NEL-M1 melanoma cells *in vitro*. The fusion protein was not toxic for chinese hamster ovary cells (CHO-K1) or green monkey kidney cells (CV-1) which do not express the α-MSH receptor. This fusion protein specifically interacts with the melanocortin receptors on the melanoma cells (Tatro *et al.*, 1992).

A conjugate of the hormone with a monoclonal antibody to CD3, an invariant component of the T cell receptor complex, was used to target melanoma cells for destruction by human cytotoxic T lymphocytes, that otherwise bear no specificity for the tumours (Liu *et al.*, 1988).

An attempt to use melphalan conjugated to α-MSH in man has been made by Lejeune and Ghanem (1993), who tested the modified drug in two patients suffering from melanoma metastases. In one case, a complete remission for more than a year was achieved and although this is not a proof that drug targeting took place, this is a promising result, and the methods could be optimised in the future. Several α-MSH conjugates containing melphalan have also been tested in a human melanoma cell line, some of which were able to specifically bind to the MSH receptor. Although their cytotoxicity was less than that of the drug alone, it was cell specific, and the effect could be inhibited by excess α-MSH (Morandini *et al.*, 1994).

The possibility of employing α-MSH derivatives in the imaging of malignant melanoma has also been investigated (Bard *et al.*, 1986; Bard *et al.*, 1990). The most recent attempt was to employ a chelating derivative of α-MSH, *bis*MSH-DTPA (diethylenamine pentaacetic acid) which appeared to be equipotent to α-MSH in the Cloudman S91 tyrosinase assay and could be labelled with ^{111}In. Tumour associated radioactivity was significantly higher than activity in other tissues, and could be inhibited by excess α-MSH (Bard *et al.*, 1990).

1.3.2. β-MSH

A greater variability is observed in the structure of β-MSH than in other MSH peptides. Some species might even express more than one form of the peptide (Lee *et al.*, 1963; Ng *et al.*, 1981). In mammals, the β-MSH sequence corresponds to

the 41-58 amino acid region of the precursor β-LPH. Most mammalian β-MSHs contain the heptapeptide Met^{7}-Glu^{8}-His^{9}-Phe^{10}-Arg^{11}-Trp^{12}-Gly^{13}, the common melanocortin sequence. H-Asp^{1}-Glu^{2}-Gly^{3}-Pro^{4}-Tyr^{5}-Arg^{6}-Met^{7}-Glu^{8}-His^{9}-Phe^{10}-Arg^{11}-Trp^{12}-Gly^{13}-Ser^{14}-Pro^{15}-Pro^{16}-Lys^{17}-Asp^{18}-OH is the most commonly found mammalian β-MSH sequence. In rats and mice, the POMC does not have a pair of basic residues between positions 59 and 60, so enzymatic cleavage between those residues might not take place, and it is not clear whether they are able to form β-MSH peptides. Systematic structure-activity studies with β-MSH peptides have not been undertaken; instead, the studies have been mainly restricted to the variety of naturally occurring analogues. In the *Rana pipiens* melanophore assay, monkey, pig and camel β-MSH are the most potent compounds exhibiting approximately 25% of the activity of α-MSH (see Eberle, 1988, p.354). Its affinity to the MSH receptor in B16 murine melanoma was found to be 20% of that of α-MSH (Eberle, 1988).

1.3.3. γ-MSH

The analysis of the nucleotide sequence of the cloned cDNA for the bovine corticotropin-β-lipotropin precursor (=POMC) showed that a section of its N-terminal region contains the His-Phe-Arg-Trp tetrapeptide found in α-MSH, β-MSH and ACTH, and was therefore named γ-MSH (Nakanishi *et al.*, 1979). It was suggested that POMC may be processed by enzymatic cleavage between pairs of basic amino acids, Arg^{-57}-Lys^{-56} and Arg^{-43}-Arg^{-42} or Lys^{-28}-Arg^{-27} (Nakanishi *et al.*, 1979), thus giving the possible fragments γ_1-MSH, γ_2-MSH and γ_3-MSH (Fig.*1.3.*).

The presence of these fragments in mammalian pituitary extracts have since been established (Böhlen *et al.*, 1981; Tanaka *et al.*, 1980). γ_1-MSH is preferentially found in the intermediate lobe of the pituitary, whereas γ_3-MSH is located in the

anterior lobe (Wolter, 1984). The existence of pro-γ-MSH containing a Lys-residue on the N-terminus has also been postulated (Bennett, 1986, Bennett *et al.*, 1986), and binding sites for Lys-γ_3-MSH were demonstrated in the rat adrenal cortex (Pedersen *et al.*, 1983). This peptide, however, was unable to stimulate membrane-associated adenylate cyclase or guanylate cyclase activity.

γ_1-MSH	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2
γ_2-MSH	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH
γ_3-MSH	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Arg-Arg-Asn-Gly-Ser-Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln-OH

Fig.1.3. Structure of γ-MSH peptides as proposed from analysis of the gene encoding the precursor POMC

γ_3-MSH peptides were subsequently detected in neurones, nerve fibres and nerve endings in the rat duodenum (Wolter, 1984). Also, γ-MSH like immunoreactive cell bodies were found in the *nucleus tractus solitarii* of the rat (Kawai *et al.*, 1984).

The exact biological function of γ-MSH peptides is not yet known, although there are reports of them being involved in numerous clinical conditions. Elevated plasma levels of γ-MSH have been associated with conditions as diverse as polycystic ovary syndrome, idiopathic hyperaldosteronism and cardiac arrest (Wortsman *et al.*, 1984; Wortsman *et al.*, 1985; Griffing *et al.*, 1985). The

presence in the *nucleus tractus solitarii* suggests that γ-MSH peptides might be involved in the regulation of the cardiovascular system. Recent results have shown that γ_2-MSH especially is active in controlling blood pressure and heart rate (Versteeg *et al.*, 1993; de Wildt *et al.*, 1994). Interestingly, opposing cardiovascular effects can be seen depending on the site of administration, a depressor effect occurring after microinjection directly into the *nucleus tractus solitarii* and a pressor response following i.v. administration.

γ_3-MSH has been shown to stimulate secretion of corticosterone and aldosterone in frog interrenal glands in a dose-dependent manner (Leboulenger *et al.*, 1986).

Within the pituitary, γ-MSH peptides were unable to evoke the release of luteinising hormone, follicle stimulating hormone prolactin, growth hormone or thyrotropin (Ling *et al.*, 1979). As a result it is unlikely that γ-MSH peptides have a role as releasing factors for anterior pituitary hormones.

γ_1-MSH, Ac-γ_1-MSH, γ_2-MSH and γ_3-MSH were tested in the *Rana pipiens* melanophore assay (Ling *et al.*, 1979). All peptides were able to stimulate the melanocytes, but only at very high concentrations compared to α-MSH. The most active peptide in this assay was Ac-γ_1-MSH, a peptide synthesised in analogy to the N-terminus of α-MSH. γ_3-MSH was the least active peptide in this study. In B16 melanoma cells, γ-MSH peptides are reported to stimulate adenylate cyclase (MacNeill *et al.*, 1981). In recent studies with Bomirski AbC1 hamster melanoma cells and Cloudman S91 murine melanoma (cell lines normally responsive to β-MSH) γ_3-MSH alone was able to trigger the biological response in the hamster cell line, but not in Cloudman melanoma cells, and only at high concentrations (10μM). However, only two concentrations (1μM and 10μM) of the peptides were tested, and it is unlikely, but possible that the peptides either exhibit activities at higher concentrations or act as partial agonists, thus not reaching the maximal response

triggered by β-MSH. All peptides were also tested for synergy with β-MSH. Whereas γ_2-MSH potentiated the effect of β-MSH when administered simultaneously, γ_3-MSH acted as a weak inhibitor of β-MSH induced tyrosinase activity (Slominski *et al.*, 1992). It was therefore proposed that this biological action could be controlled by the C-terminal part of the peptide.

Receptor binding of γ-MSH peptides has not been studied in melanocytes or melanoma cells.

1.3.4. ACTH

ACTH is not strictly speaking a melanotropic peptide, but it derives from the same precursor molecule, POMC, and because of its structural similarity to MSH (the first 13 amino acids of ACTH form α-MSH) it is able to evoke most of the actions of α-MSH with a lower potency. ACTH will interact with the α-MSH receptor, and shows approximately 10% of the affinity of α-MSH to the MC1 receptor in melanoma cells (Eberle, 1988).

1.4. Melanocortin Receptors

Melanocortin receptors are a family of G protein-coupled receptors. They occur mainly in the central nervous system and in melanocytes. Molecular cloning of several receptors belonging to sub-families of melanocortin receptors has recently been reported (Mountjoy *et al.*, 1992; Chhajlani and Wikberg, 1992; Gantz *et al.*, 1993a,b; Roselli-Rehfuss *et al.*, 1993; Chhajlani *et al.*, 1993), and the sequences now available allow new insights into the interaction of melanotropic hormones with their receptors. Melanocortin receptors show features that are quite different from other neuropeptide receptors: they lack a number of amino acids usually found in G protein-coupled receptors and they also show a different

transmembrane topology with the second extracellular loop being very hydrophobic. It has therefore been suggested that no significant extracellular domain exists in this part of the receptor (Cone and Mountjoy, 1993).

1.4.1. MC1-Receptors

The MC1 receptor can be found in melanocytes and melanoma cells and is undoubtedly the most widely investigated of all melanocortin receptors. The amino acid sequence for the human and murine MC1 has been independently reported by several groups (Mountjoy *et al.*, 1992; Chhajlani and Wikberg, 1992; Gantz *et al.*, 1993a). Whereas the human receptors described by Mountjoy *et al.* and Gantz *et al.* were identical, the one reported by Chhajlani and Wikberg differs by two amino acids. The murine and human MC1 receptors are largely identical (Mountjoy *et al.*, 1992).

Chhajlani and Wikberg used competitive binding experiments in COS-7 cells transfected with the human MC1 receptor to distinguish between ligand affinities to this receptor. Several melanotropic peptides were able to displace [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH in the following order: [Nle^4,D-Phe^7]α-MSH > α-MSH > ACTH > β-MSH > γ-MSH > $ACTH_{(4-10)}$. The non-melanotropic POMC peptide β-endorphin could not displace the radioactive ligand from the receptor.

Mountjoy *et al.* reported data from the cAMP assay for the receptor cloned from Cloudman S91 mouse melanoma: [Nle^4,D-Phe^7]α-MSH is the most potent ligand, followed by α-MSH and β-MSH with similar activities and ACTH with a slighter lower activity. γ-MSH did not exhibit any biological activity (Mountjoy *et al.*, 1992).

The MSH receptor from B16 murine melanoma cells has been characterised by photoaffinity labelling with a photoreactive mono-iodinated derivative of α-MSH, [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}, ATB-Lys^{11}]α-MSH (Ahmed *et al.*, 1992). After isolation of the ligand-receptor complex, SDS-PAGE and subsequent autoradiography, it appears as a doublet band of 43-46kDa. After deglycosylation, the receptor shows a single band of approximately 28kDa (Ahmed *et al.*, 1993), which suggests that the receptor exists in two very similar forms, which might vary in their glycosylation status. Several other groups came to similar results: Gerst *et al.* (1988) reported the presence of two receptor proteins of 43-46kDa on B16-M2R melanoma cells. Solca *et al.* (1989) found a hormone receptor complex of 45kDa on a panel of human and murine melanoma cells including the B16 and Cloudman S91 cell lines and later described the existence of three different receptor subtypes on various B16 mouse melanoma subclones (Solca *et al.*, 1991).

Recently, a MC1-type receptor isolated from primary rat Schwann cells has been reported (Dyer *et al.*, 1993). This is the first receptor of this type that does not origin from melanocytes or melanoma cells. [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7},ATB-Lys^{11}]α-MSH photoaffinity labelled proteins were characterised by SDS-PAGE followed by autoradiography and show a doublet band of 42-45kDa similar to those observed in B16 murine melanoma cells (Ahmed *et al.*, 1992).

1.4.2. MC2-Receptor

In situ hybridisation shows that the MC2 receptor is localised in the adrenal cortex, mainly in the *zona fasciculata*, where glucocorticoids are produced and in the cortical half of the *zona glomerulosa*, the site of aldosterone production (Mountjoy *et al.*, 1992). It has been proven to be specific for ACTH by Northern blot analysis, and Cloudman S91 melanoma cells transfected with the gene for the MC2 expression respond to stimulation with ACTH peptides.

1.4.3. MC3-Receptors

Molecular cloning of the rat MC3 receptor has been reported from the hypothalamus, and it also occurs in the limbic system (Roselli-Rehfuss *et al.*, 1993). It contains 323 amino acids and is 43% identical to the MSH-R from melanocytes. In cells transfected with this receptor, [Nle^4,D-Phe^7]α-MSH stimulates cAMP synthesis most, followed by α-MSH, γ_1-MSH, γ_2-MSH and ACTH, which are equipotent. γ_3-MSH, desacetyl-α-MSH and $ACTH_{(1-10)}$ are considerably less active and Org 2766 does not exhibit any activity (Roselli-Rehfuss *et al.*, 1993).

The human equivalent consists of 363 amino acids and can be found in the brain and in gut and placental tissues, but not in melanocytes or the adrenal gland (Gantz *et al.*, 1993a). Adenylyl cyclase is activated equally well by α-MSH, β-MSH and γ-MSH and $ACTH_{(1-39)}$, but to a lesser extent by $ACTH_{(1-10)}$ and $ACTH_{(4-10)}$.

Roselli-Rehfuss and co-workers suggest the possibility that this receptor might have a certain specificity for γ-MSH peptides, although their physiological role is not yet understood. Gantz *et al.*, however, propose that the receptor recognises the nearly completely conserved core sequence of all melanocortin peptides, $ACTH_{(4-10)}$.

Photoaffinity labelling of this receptor shows a single band at approximately 53kDa, which is reduced to 36kDa after enzymatic deglycosylation (Quarawi, personal communication).

1.4.4. MC4-Receptors

The human MC4 receptor is expressed in the brain, but not in melanocytes, the adrenal cortex or the placenta (Gantz *et al.*, 1993b). It responds equally to α-MSH, α-MSH and ACTH, but less well to γ-MSH, $ACTH_{(1-10)}$ and $ACTH_{(4-10)}$. It was suggested that it might require Tyr^2 and Pro^{12} for stimulation of the biological activity. The physiological role of this receptor is not yet known.

1.4.5. Other melanocortin receptors

At least one other melanocortin receptor has been isolated (Chhajlani *et al.*, 1993), and has also been assigned the name MC2 by its authors. Following the nomenclature practised by Cone *et al.* as well as Gantz *et al.*, this would be a fifth melanocortin receptor. This receptor is expressed in the brain, but is not present in melanoma cells. This receptor shows a very high affinity to [Nle^4,D-Phe^7]α-MSH, followed by much lower binding of α-MSH and ACTH, which are equipotent, then by β-MSH and γ-MSH. It is likely that in the future, several other melanocortin receptors will be detected in various tissues, and from various species.

1.5. Objectives

The work presented in this thesis was carried out as part of a research programme in selective drug delivery and targeting. The chosen model peptide, α-MSH acts on pigment cells, and was therefore selected as a putative carrier for the selective treatment of malignant melanoma. In spite of the clinical relevance of this model, this project was concerned with the fundamental understanding of receptor-ligand interaction and was aimed at evaluating the binding and activity properties of the α-MSH molecule. This has been attempted by systematically investigating the influence of individual amino acids and groups of residues on the peptide's

interaction with melanocortin receptors, namely a MC1 receptor from B16 murine melanoma cells and a MC3 receptor from rat hypothalamus. An attempt was made to investigate the bioactive conformation of the molecule. Most of this study was focused on the MC1 receptor as it is relatively specific for α-MSH and its physiological functions are well established. The availability of a range of melanocortin receptor amino acid sequences and the existence of cell clones expressing large numbers of receptors isolated from the brain (e.g. the MC3 receptor) has rendered it possible to investigate the interaction of the hormone with those receptors that provide a putative site for mediating CNS side-effects if α-MSH were to be used in drug targeting.

Terminal deletion fragments have been synthesised and tested for their binding affinity and biological activity in B16 murine melanoma cells. Several of these peptides have previously been investigated by other groups, yet many of these studies were carried out using reptilian bioassays and a systematic study has not been attempted in mammalian melanoma cells. An alanine scan was performed throughout the molecule to investigate the importance of residues within the peptide core. This technique has previously been employed to identify the amino acid side-chains involved in peptide hormone-receptor-interactions while leaving the stereochemistry of the backbone unchanged. Alanine substituted analogues of α-MSH were tested for receptor binding on the MC1 and MC3 receptor and for biological activity on the MC1 receptor. Derivatives of [Nle^4,D-Phe^7]α-MSH were tested for receptor binding and activity on the MC1 receptor as [Nle^4,D-Phe^7]α-MSH is known to be a potent analogue of α-MSH. Replacement by D-amino acids might stabilise a reverse or β-turn conformation of the peptide core backbone, which has previously been proposed. A cyclic α-MSH derivative and its linear analogue were studied in order to obtain information about the active conformation of the molecule.

Reference peptides used in this study were α-MSH as the native ligand for the receptor on B16 murine melanoma cells, [Nle^4,D-Phe^7]α-MSH as a "superpotent", more stable analogue, employed as an internal standard in all assays, and γ_1-MSH as the putative ligand for the MC3 receptor. γ_1-MSH contains all the crucial amino acids from the α-MSH core sequence (α-MSH$_{(4-9)}$) and a possible specific physiological role for γ-MSH peptides has been proposed on the MC3-R.

This study provides insight into the molecular mechanism of interaction between, α-MSH, and its receptors from peripheral tissues and the CNS. Residues participating in receptor-ligand interaction could be clearly identified, and the different requirements of the two receptors was studied.

CHAPTER 2

Materials and Methods

2.1. Cell Culture

2.1.1. Solutions

2.1.1.1. Water

Water for preparation of cell culture media and solutions was freshly double glass distilled using a bi-distillation Fistreem still (Fisons Ltd.) fitted with a Fistreem predeionizer (Fisons Ltd.)

2.1.1.2. Balanced salt solutions

Phosphate buffered saline (PBS) without divalent cations was obtained from Oxoid Ltd in tablet form. One tablet was dissolved in 100ml of freshly double distilled water and autoclaved when required. Hank's Balanced Salt Solution and Dulbecco 'B' were bought from Gibco as 10X liquid and diluted aseptically with freshly double distilled water. Solutions were stored at 4^{o}C for up to four weeks.

2.1.1.3. Base and acid solutions

Solutions of 7.5% w/v sodium bicarbonate and 1M sodium hydroxide were prepared using double distilled water and were steam sterilised. 0.1mM HCl was prepared by diluting 1M HCl (BDH Laboratory Reagents Ltd.) in double distilled water followed by filter sterilisation.

2.1.1.4. Ethylenediaminetetraacetic acid (EDTA)

A 0.02% w/v solution of the disodium salt (BDH Laboratory Reagents Ltd.) was prepared in PBS, sterilised and stored at -20°C for up to 4 months.

2.1.1.5. Trypan blue

The stain was obtained from BDH Laboratory Reagents Ltd. and dissolved at 0.1% w/v in PBS.

2.1.1.6. Growth media and additives

All media and additives except some batches of foetal calf serum (FCS) were purchased from Gibco, Paisley. RPMI 1640 medium and Minimum Essential Medium (MEM) Eagle with Earle's salts were obtained as 10X concentrates without L-glutamine or sodium bicarbonate but containing phenol red. L-glutamine (200mM), a solution of penicillin (5000IU/ml) and streptomycin (5000mg/ml), non-essential amino acids and MEM vitamin solution were obtained as 100X concentrates and stored at the appropriate temperature.

FCS batches were tested for the support of cell growth before use. Batches used were 30A0212S (Gibco), 10855 (Flow) and 30F30315 (Gibco). It was added to the growth medium at a concentration of 10%.

Growth media were prepared aseptically according to the following recipe, stored at 4°C and used within a week of preparation. If required, the medium was adjusted to a final pH of 7.2-7.4 with 1M NaOH or 1M HCl.

	RPMI 1640	MEM Eagle
10X RPMI 1640	50ml	
10X MEM Eagle		50ml
L-glutamine	5ml	5ml
penicillin/streptomycin	5ml	5ml
MEM non-essential amino acids	5ml	5ml
MEM vitamin solution		7.5ml
NaHCO3	13.5ml	17.5ml
foetal calf serum	55ml	55ml
water	421.5ml	415ml

2.1.2. Equipment

2.1.2.1. Laboratory equipment

A laminar flow cabinet (MDH Ltd.) with vertical recirculation was used when aseptic techniques were required. Cells were maintained in a LEEC PF2 anhydric incubator (Laboratory and Engineering Company) with forced air circulation at 37°C. The temperature control of the incubator was checked regularly. An inverted biological microscope, WILD M40 (Wild Heerbrugg Ltd) was used for examining cell growth and density. A standard double grid haemocytometer (Fisons Ltd.) was employed for determining cell numbers.

2.1.2.2. Disposable Items

Sterile tissue culture polystyrene flasks were regularly obtained from Falcon, Becton and Dickinson and Co.. 24- and 96-well plates were obtained from Nunc.

Sterile screw capped tubes were obtained from Sterilin Ltd. Polypropylene ampoules (Corning) were used for storage of cells in liquid nitrogen.

2.1.2.3. Glassware

All glassware was rinsed in tap water immediately after use and then soaked in a 2% solution of RBS 25 (Fisons Ltd.) for 30 min. Articles were subsequently rinsed in three changes of tap water and distilled water. Finally, items were left in double distilled water for 1h, dried in an hot air oven (Gallenkamp), capped with aluminium foil and sterilised by dry heat at 160°C for 1h (Gallenkamp sterilising oven).

2.1.3. Cell Culture Methods

2.1.3.1. Cell lines

A B16 murine melanoma cell line was donated by L. R. Kelland, Institute of Cancer Research, Sutton. Human 293 cells transfected with the genes for the expression of the MC3 receptor were supplied by R.D. Cone, Vollum Institute, Portland, Oregon.

2.1.3.2. Cell line maintenance and subculture

Cells from both cell lines were maintained in a LEEC anhydric incubator at 37°C under standard conditions of 95% air/5% CO_2 in a humidified atmosphere. Cultures were examined daily for microbial contamination. In order to maintain a pH of 7.2-7.4, the medium was routinely changed the day before the cells reached confluence. Subculture was undertaken with confluent cells by washing the cell monolayer twice with PBS and then incubating with a sufficient volume of 0.02% EDTA/PBS for 10min. Detached cells were then diluted with culture medium to 10ml, a sample taken and counted and flasks were inoculated with $2x10^6$ cells per 175cm^2.

Cells used in the melanin biosynthesis assay were grown in MEM Eagle medium. The same routine was followed to maintain cultures for this assay.

2.1.3.3. Determination of cell density

After detaching the cells from the flasks, a 0.4ml sample of the cell suspension was mixed with 0.1ml trypan blue solution. Viable cells exclude the dye, whereas non-viable cells are stained dark blue. The cells-dye mixture was then loaded onto a grid haemocytometer under a coverslip. A count of the four corners and the central square was made with an inverted microscope, then the number of cells was calculated using the following equation:

$$\text{cells/ml} = (\text{total cells in 5 chambers} \times 10^4)/4$$

2.1.3.4. Cell storage and recovery

Cells were stored in 2ml ampoules in the vapour phase of a Union Carbide LR-40 liquid nitrogen refrigerator at approximately -148°C. Cells were prepared for storage by detaching confluent cells from the flasks using the method described in section 2.1.3.2, suspending them in culture medium, centrifuging them for 10 minutes at 1000 rpm and resuspending them in culture medium containing 10% dimethyl sulphoxide (Aldrich, spectrophotometric grade) as a cryoprotectant. Ampoules were then placed in a Union Carbide BF6 biological freezer unit plug and put in a Union Carbide LR-33 liquid nitrogen refrigerator to allow them to cool below -70°C at a rate of approximately 1°C min^{-1}.They were then transferred to the liquid nitrogen refrigerator for long term storage.

To recover cells from storage, the cryotubes were placed in a 37°C water bath and thawed. Cells were then suspended in 10ml of culture medium, centrifuged for 10 min at 1000rpm, resuspended in 75ml of prewarmed medium and transferred into a 175cm^2 flask.

2.2. Peptide Synthesis

All peptides except α-MSH, Ac-α-$MSH_{(1-12)}$-NH_2, Ac-α-$MSH_{(1-11)}$-NH_2 and Ac-α-$MSH_{(1-10)}$-NH_2 were synthesised by Dr. G.W.J. Olivier, and their preparation is therefore not described in detail. γ-MSH, α-MSH-COOH and [Cys^4,Cys^{10}]α-$MSH_{(4-13)}$ were obtained commercially from Bachem, Basel, Switzerland. FAB-MS was carried out at Swansea University SERC Mass Spectrometry Service and MALDI-TOF MS by Kratos Analytical. Two Jeol FAB-MS spectra were provided by Dr.J. Thomas-Oates at the Mass Spectroscopy Centre, University of Utrecht.

2.2.1. Reagents

Amino acid derivatives, polydimethylacrylamide-Kieselguhr resin (Pepsyn K) and *p*-[*R,S*-α-1(9*H*-fluoren-9-yl)methoxy-formamido-2,4-dimethoxybenzyl]phenoxy-acetic acid (AM-linker) were obtained from MilliGen, Watford. Hydroxybenzotriazole (HOBT), trifluoroacetic acid (TFA), diisopropyl-carbodiimide (DIC), 1,2-ethanedithiol (EDT), anisole and phenol were purchased from Aldrich, Gillingham, Dorset. Analytical and semi-preparative HPLC-columns were packed with Techsphere 10μm ODSWP(PTS 031 WP 300) as stationary phase (HPLC Technology, Macclesfield). Preparative HPLC was carried out using a C18 protein-peptide column 25cm x 2.5cm i.d.. All other reagents were of analytical grade.

2.2.2. Synthesis of α-MSH, [Nle^4,D-Phe^7]α-MSH and α-MSH fragments

All peptides, except Ac-α-$MSH_{(1-10)}$-NH_2, for which a semiautomatic CRB-Pepsynthesiser II was used, were assembled on a MilliGen 9050 fully automatic synthesizer. They were prepared as their N-acetyl carboxyamide form by solid-

phase synthesis using Fmoc-strategy (Atherton and Sheppard, 1989). Serine was used as the 3,4-dihydro-4-oxobenzotriazin-3-ylester, all other amino acids as pentafluorophenyl esters. Side-chain protecting groups were used as follows: arginine, methoxytrimethylbenzenesulphonyl (Mtr); glutamic acid, *t*-butoxy (OBut); histidine, *t*-butoxycarbonyl (Boc); lysine, (Boc); serine, *t*-butyl (But); tyrosine, (But). A four-fold molar excess of reagents was used at each coupling. Ac-α-$MSH_{(1-10)}$-NH_2, Ac-α-$MSH_{(1-11)}$-NH_2, Ac-α-$MSH_{(1-12)}$-NH_2 and α-MSH were deprotected and cleaved from the resin using 2% EDT, 2% anisole and 2% phenol in TFA overnight at room temperature. For Ac-α-$MSH_{(2-13)}$-NH_2, Ac-α-$MSH_{(3-13)}$-NH_2, Ac-α-$MSH_{(4-13)}$-$NH_2$and Ac-α-$MSH_{(5-13)}$-NH_2, 2.5% anisole and 2.5% EDT in TFA were used. Deprotection and cleavage of [Nle^4,D-Phe^7]α-MSH employed 2% EDT, 2% anisole and 1% water in TFA. Peptides were purified by preparative HPLC using a gradient elution of 0.1% TFA in water and 0.1% TFA in 90% acetonitrile/10% water at 10ml/min. The eluent was monitored by UV spectrophotometry at 217 nm. Fractions were collected at 30s intervals and tested by analytical scale HPLC. Peptide identities were confirmed by FAB-MS or MALDI-TOF MS: α-MSH M+H calc. 1664.8, found 1664; [Nle^4, D-Phe^7]α-MSH M+H calc. 1646.8, found 1647; Ac-α-$MSH_{(1-10)}$-NH_2 M+H calc. 1340.6, found 1341; Ac-α-$MSH_{(1-11)}$-NH_2 M+H calc. 1468.7, found 1469; Ac-α-$MSH_{(1-12)}$-NH_2 M+H calc. 1565.7, found 1566; Ac-α-$MSH_{(2-13)}$-NH_2 M+H calc. 1577.8, found 1578; Ac-α-$MSH_{(3-13)}$-NH_2 M+H calc. 1414.7, found 1415; Ac-α-$MSH_{(4-13)}$-NH_2 M+H calc. 1327.7, found 1327; Ac-α-$MSH_{(5-13)}$-NH_2 M+H calc. 1196.6, found 1197.

2.2.3. Synthesis of alanine-substituted analogues of α-MSH

Alanine analogues of α-MSH were synthesised in two parallel assemblies on the MilliGen 9050 automatic synthesizer. Fmoc amino acids were activated prior to

reaction with O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU).

Side chain protecting groups were used as described above. Peptides were deprotected and cleaved from the resin using a mixture of 94% TFA, 2% anisole, 2% EDT and 2% phenol and purified as before. Peptide identity was confirmed by FAB-MS or MALDI-TOF MS: [Ala^1]α-MSH M+H calc. 1648.8, found 1649; [Ala^2]α-MSH M+H calc. 1572.8, found 1573; [Ala^3]α-MSH M+H calc. 1648.8, found 1650; [Ala^4]α-MSH M+H calc. 1604.8, found 1605; [Ala^5]α-MSH M+H calc. 1606.8, found 1606.4; [Ala^6]α-MSH M+H calc. 1598.8, found 1599; [Ala^7]α-MSH M+H calc. 1588.8, found 1589; [Ala^8]α-MSH M+H calc. 1579.7, found 1579.5; [Ala^9]α-MSH M+H calc. 1549.8, found 1551; [Ala^{10}]α-MSH M+H calc. 1678.8, found 1679; [Ala^{11}]α-MSH M+H calc. 1607.7, found 1608; [Ala^{12}]α-MSH M+H calc. 1638.8, found 1639; [Ala^{13}]α-MSH M+H calc. 1636.8, found 1637 (Sahm *et al.*, 1994).

2.2.4. Synthesis and cyclisation of [Cys^4,Cys^{10}]α-MSH

Synthesis was carried out as described above; the peptide was deprotected and cleaved from the resin using a mixture of 95% TFA, 2.5% EDT and 2.5% anisole. For the cyclisation, 100mg of the crude peptide was dissolved in 3g of glacial acetic acid in a 500ml round bottom flask, the mixture was then degassed using N_2. Approximately 250ml of degassed water was then added and the solution was titrated to pH 7.5 with NH_4OH in a nitrogen atmosphere. The flask was filled to the neck with degassed water and the mixture was then exposed to air to allow the oxygen to dissolve slowly and oxidise the peptide until the cyclisation was complete. To test for cyclisation, the solution was co-chromatographed with the pure linear peptide as there are only marginal changes in retention time between the linear and cyclised form of the peptide. To separate the cyclised compound from the buffer, the solution was adjusted to contain 3.5% acetonitrile and passed

through a C18 reverse phase Bond Elut column packed with Spherisorb ODS (Analytichem International). The column was washed with 3.5% acetonitrile to remove the salt. The peptide adsorbed to the column was then washed off with an acetonitrile-water mixture, lyophilised and purified by HPLC The washings were tested for absence of peptide by HPLC and further extractions using the Bond Elut column were performed until no trace of peptide was found in the buffer solution. Identity of the cyclised peptide was confirmed by FAB-MS: [$\overline{Cys^4,Cys^{10}}$]α-MSH M+H calc. 1681.74, found 1682.

2.2.5. Synthesis of [Nle^4,Asp^5,D-Phe^7, Lys^{10}, Arg^{11}]α-MSH

Synthesis was performed and purification was carried out as described in section *2.2.2.* using pentafluorophenyl esters of the amino acids. Peptide identity was confirmed by FAB-MS: M+H calc. 1731.91, found 1733.

2.2.6. Synthesis of [Nle^4,D-Phe^7,D-Trp^9]α-MSH

Synthesis and purification were carried out as described, using TBTU activation. Identity of the peptide was confirmed by FAB-MS: M+H calc 1646.8, found 1646.5.

2.2.7. Synthesis and cyclisation of [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH

The peptide was assembled using TBTU activation as described above but using Fmoc-Asp(OAll)-OH for position 5 and Fmoc-Lys(Aloc)-OH for position 10. On the Pepsynthesizer II, protecting groups in positions 5 and 10 were then removed by recirculating for 2h with palladium-tetrakis-triphenyl phosphene in a mixture of 5% glacial acetic acid and 2.5% *N*-methyl morpholine in chloroform under exclusion of air. The resin was the washed with 0.5% diethyl-dithiocarbamate and

0.5% DIPEA in DMF to remove the palladium complex, then with DMF. Cyclisation was started by activation with TBTU and ended when no free amino groups could be found. This method was first described by Kates *et al.* (1993). Deprotection and cleavage from the resin was carried out by reacting with 5% thioanisole, 3% EDT and 2% anisole in TFA for 16h at room temperature. The peptide was then purified by HPLC as described. FAB-MS revealed that this peptide was not cyclised, but was likely to still contain the Aloc-protection group on the Lys^{10}. M+H calc. 1685.88, found 1783.9.

2.3. Radioiodination of [Nle^4,D-Phe^7]α-MSH

[Nle^4,D-Phe^7]α-MSH has been iodinated following a methodology described by Eberle (1988).

2.3.1. Solutions

The following solutions were prepared either immediately before use or stored at 4°C where appropriate:

1) 0.25M Na_2HPO_4 (FSA Laboratory supplies)
2) 0.25M NaH_2PO_4
3) 1%TFA (Aldrich, analytical grade)
4) 50, 60 and 80% methanol containing 1% TFA
5) 0.25M phosphate buffer pH 7.4
6) 0.25% BSA in 0.05M phosphate buffer pH 7.4
7) 1% Polypep (Sigma) in 0.05M phophate buffer pH 7.4
8) 0.1% chloramine T (BDH Chemicals Ltd.) dissolved in water immediately prior to use

2.3.2. Preconditioning of the Bond-Elut column

A C18 reverse phase 'Bond Elut' column packed with Spherisorb ODS was preconditioned by washing it in the following way:

3x1ml 1% TFA

3x1ml 80% MeOH/1%TFA

1x1ml Polypep solution

3x1ml 80%MeOH/1%TFA

3x1ml 1% TFA

2.3.3. Iodination

1.5µl of [Nle^4,D-Phe^7]α-MSH stock solution was diluted in 20µl of phosphate buffer. 10µl of $Na^{125}I$ solution and 10µl of chloramine-T solution were then added and allowed to react for 30s before addition of 0.6ml BSA solution. The reaction mixture was then applied to the preconditioned Bond-Elut column to remove free $Na^{125}I$. The column was washed twice with phosphate buffer, four times with 50% methanol/1% TFA and twice with 60% methanol/1% TFA. The methanolic washes were then injected onto an analytical grade HPLC for further purification.

2.3.4. Purification

The methanolic fractions containing the iodinated peptides were purified by analytical scale HPLC using an exponential gradient of 0.1% TFA in water and 0.1% TFA in 70% ACN/30% water. Fractions were collected at 1min intervals from 25 to 40 minutes after injection. The moniodinated peptide eluted before the di-iodinated derivative, but after the free [Nle^4,D-Phe^7]α-MSH (Fig. 2.1.). The

radioactivity of the fractions was then counted, and those with radioactivity associated with the peak of the monoiodinated peptide pooled and their activity counted.

The radiolabelled peptide could be stored at -20°C for up to 20 days.

2.3.5. Calculation of radiotracer concentration

The concentration of radiotracer was calculated from the measured radioactivity by the following method:

Assuming a 1:1 ratio of ^{125}I to [Nle^4,D-Phe^7]α-MSH (mono-iodinated [Nle^4,D-Phe^7]α-MSH by definition):

1matom $^{125}I \equiv 10^{-3}$ moles [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

and with a specific activity of 80.5×10^{12}Bq/matom for carrier-free Na^{125}I,
1mole [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH would have an activity of $80.5 \times 10^{12} \times 10^3$Bq

As 1Bq=1decay per second=60 decays per minute and the effiency of the gamma-counter=70%:

1mole [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH would register $80.5 \times 10^{12} \times 10^3 \times 60 \times \frac{70}{100}$
$= 3.38 \times 10^{18}$ cpm on the LKB 1277 Gammamaster.

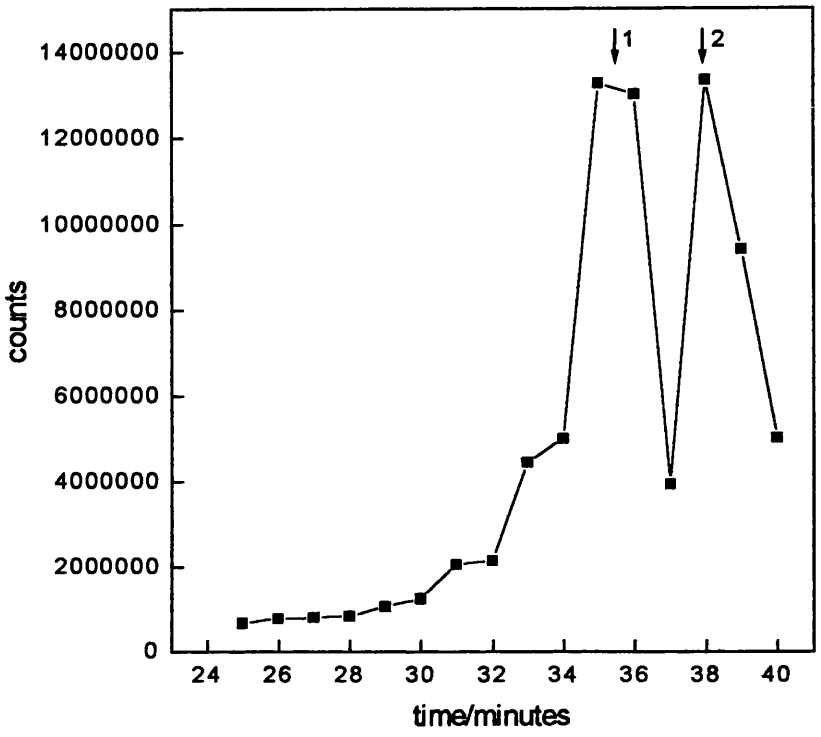


Fig. 2.1. Elution profile of mono- and di-iodinated α-MSH from analytical scale HPLC as described in section *2.3.4.* Mono-iodinated α-MSH (1) elutes at 35min, di-iodinated (2) α-MSH at 38min

2.4. Binding Assays

Binding assays were carried out following a method adapted from Siegrist *et al.* (1988) as described by Erskine-Grout (1993).

2.4.1. Binding medium

The binding medium consisted of RPMI 1640 medium without any additives, 25mM N-(2-hydroxyethyl)piperazine-*N'*-2-ethane sulphonic acid (HEPES) and 0.2% BSA. HEPES and BSA were prepared as 10X concentrates in serum-free RPMI 1640, stored at -20°C and diluted immediately prior to the experiments.

2.4.2. Binding isotherm of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

Cells were detached from the flasks, counted and seeded at a density of $5x10^5$ cells per well into 24-well plates. They were then incubated under standard conditions for 15h before being washed twice with ice-cold RPMI 1640 without additives and allowed to cool to 0-4°C while the binding buffer was being prepared. Various concentrations of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH were added to the medium. Non-specific binding was determined using a 1000-fold excess of non-labelled [Nle^4,D-Phe^7]α-MSH. 0.5ml of binding medium containing the radiotracer was added to the wells and the cells were incubated for 8h at 0-4°C. After the incubation, excess radiolabelled peptide was washed off with RPMI 1640, the cells lysed with 1M NaOH and the radioactivity counted on a LKB 1277 gammamaster gamma-counter. Estimates for receptor number and association constant were obtained using a non-linear least square regression suitable for ligand binding analysis (see 2.4.2.1.) A Scatchard plot was carried out to prove the homogeneity of the receptor population (Scatchard, 1949).

2.4.2.1. Scatchard plot

Assuming equilibrium is reached between free and occupied receptors, the Law of Mass Action can be used to describe this equilibrium:

$$[H][R] \underset{Kd}{\overset{Ka}{\rightleftharpoons}} [HR]$$

with: [H] = concentration of free hormone

[R] = concentration of free receptor

[HR] = concentration of hormone-receptor complex

if n = total number of receptors,

$$[R] = n - [HR]$$

and

$$K_a \equiv \frac{[HR]}{[H]\,(n-[HR])}$$

combination of these two equations and rearrangement into the linear form gives:

$$\frac{[HR]}{[H]} = K_a\ (n-[HR])$$

where [HR]/[H] is the y-axis, [HR] the x-axis, the slope = $-K_a$ and the x-intercept = n (Scatchard, 1949).

2.4.3. Competition binding

Competition binding was studied using the assay protocol for the isotherm described in section 2.4.2., but with the binding buffer containing a fixed concentration of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH, nominally 0.1nM and serial dilutions of the test peptide, usually in the range of 10^{-6} to 10^{-12} M.

2.4.3.1. Analysis of binding data

MINSQ non-linear least square regression analysis was used to calculate dissociation constants from the competition binding data employing the following equation:

$$\text{cpm(test)} = \frac{\text{cpm}_{max} - \text{cpm}_{min} \times [A]}{[A] + K_{dA} \times \left(\frac{[B]}{K_{dB}}\right)} + \text{cpm}_{min}$$

with cpm(test) = cpm for sample point

cpm_{max} = maximum bound (no competitor)

cpm_{min} = minimum bound (excess competitor)

[A] = conc. of tracer

[B] = conc. of competitor

K_{dA} and K_{dB} = dissociation constant of tracer and competitor, respectively

2.4.4. Binding to human 293 cells transfected with the gene encoding the MC3 receptor

Preliminary binding studies with the cells transfected with the gene that encodes the rat hypothalamus receptor were carried out at 37°C and 0-4°C. Time-course experiments at 0-4°C showed that equilibrium binding was reached after 24h (see Fig. *5.2.*). A monolayer could not be maintained in 24-well plates at 0-4°C for

such a long period of time, so the experiments were carried out in 96-well filtration plates (nitro-cellulose filters, Millipore). The plates are made of a solvent resistant plastic with polyvinylidene difluoride sonically welded to the bottom of each plate which is removed after the incubation before counting the cell-associated radioactivity. These filter plates allow the washing of the cells to be carried out using a vacuum-manifold and detached cells are not lost during the washing procedure. The plates were inoculated with 10^5 cells per well and incubated for 4h at 37°C. After washing the cells three times with cold serum-free RPMI 1640 or PBS using the vacuum-manifold, 0.1ml of the binding buffer was added and a 24h incubation at 0-4°C followed. After the incubation, the cells were washed three times and the filters with the cells transferred to LP4 tubes using the Multiscreen Assay System (Millipore) after removing the polyvinylidene difluoride film from the plates. Control experiments with B16 cells were carried out to ensure that the multiscreen assay conditions did not affect the results.

2.5. Tyrosinase Activity Assays

The tyrosinase assay was based on a method described by Pomerantz (1966).

2.5.1. Assay medium

The binding medium consisted of RPMI 1640 containing 0.2μCi/ml of [3',5'-^{3}H]-L-tyrosine (Amersham). The radiolabelled amino acid was stored at 2-5°C and was added to the medium on the day of the experiment.

2.5.2. ^3H_2O standard curve

The standard curve was established using ^3H_2O internal standards from Pharmacia (Internal standard kit for liquid scintillation counting ^3H in organic solvents, 0.0909μCi=3.36kBq) on a LKB Wallac 1215 Rackbeta liquid scintillation counter (Erskine-Grout, 1993)

2.5.3. Assay procedure

The cells were seeded in 24-well plates at a density of $3x10^4$ cells/well and incubated under standard conditions for 15h. The medium was then replaced with assay medium containing serial dilutions of the test peptides and the plates were incubated at 37°C in 5%CO_2 for 48h. 0.9ml of the medium was then pipetted off into Eppendorf tubes and 0.2ml of a suspension of 10% dried charcoal in PBS was added. The mixture was agitated for 30min at 4°C and then centrifuged for 15min at 3500g (Erskine-Grout, 1993). 0.9 ml of the mixture was then added to 8ml of liquid scintillation fluid (Optiphase Safe, Pharmacia) and the radioactivity was counted using parameters matching the standard curve. To account for non-enzymatically generated ^3H_2O, wells were incubated without cells in the assay medium under assay conditions and values were subtracted from the counts. EC_{50} values were estimated using linear regression for the linear part of the dose-response curve.

2.5.3.1. Calculation of EC_{50} values

INSTAT linear regression analysis was performed on the linear part of the curve which can be described by the following equation:

$$dpm = M \log[A] + c \quad \text{or} \quad \log[A] = \frac{dpm - c}{M}$$

where dpm = ^3H_2O formation, measured as dpm

log[A] = log concentration of agonist

M = slope of the line

c = y-axis intercept

As the EC_{50} is the point of the curve where 50% of maximum stimulation is reached,

$$EC_{50} = \text{antilog} \left(\frac{(dpm_{min} + 0.5(dpm_{max} - dpm_{min}) - c)}{M} \right)$$

2.6. Melanin Assays

2.6.1. Assay procedure

The melanin biosynthesis assay was carried out following a procedure first described by Siegrist and Eberle (1986). For this assay, it was necessary to grow the cells in MEM Eagle with Earle's salts as melanin is not as readily soluble in RPMI 1640. Cells were plated in 96-well plates at a density of 2.5×10^3 cells per well. After a 15h incubation under standard conditions, serial dilutions of the peptide were added in MEM medium containing 0.3mM L-tyrosine (Sigma) to serve as a substrate for melanin biosynthesis. After a 72h incubation at 37°C,

melanin biosynthesis was measured spectrophotometrically on a Microplate reader (ICN/Flow) using 340nm and 405nm filters. A standard curve obtained with synthetic melanin (Sigma) was linear under the conditions of the experiment (Fig. 2.2.). Although the absorbance observed at 340nm was higher than at 405nm, the 405nm filter was used routinely because the medium shows a relatively high absorbance at 340nm itself and readings at this wavelength repeatedly deviated from the linear range of the spectrophotometer (A>1.0).

2.6.1.1. Calculation of EC_{50} values

The calculation of EC_{50} values was performed as described in section *2.5.3.1.*, but using absorbance values instead of dpm.

2.7. Statistical data analysis

Significant differences were determined from the data with a one way analysis of variance following Fisher's multiple comparison procedure at the 99% confidence interval unless otherwise stated. The analysis was carried out using *MINITAB 9.1.* The coefficient of variance associated with the four replicates wells for each concentration point in any of the assays was less than 10%. Results of the statistical analysis of the data is shown in Appendix 1.

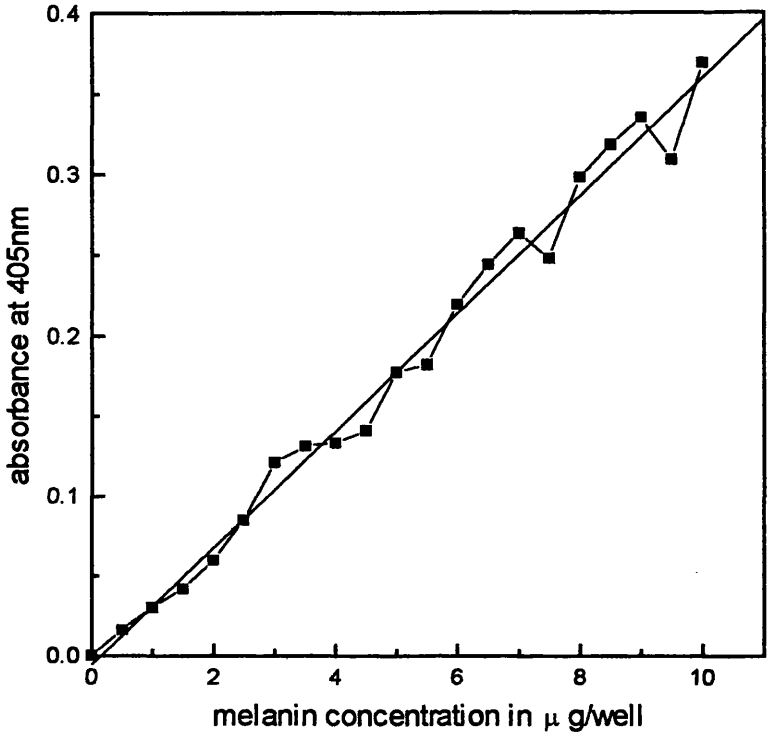


Fig. 2.2. Standard curve obtained with synthetic melanin at 405nm

CHAPTER 3

Results

Binding affinity and Biological Activity of α-MSH Derivatives in B16 Mouse Melanoma

This section describes the results obtained with the MC1 receptor from B16 murine melanoma cells in culture. Several groups of MSH peptides have been tested for receptor binding (see section *2.4.*) and tyrosinase stimulating activity, some of them also for stimulation of melanin biosynthesis (see sections *2.5.* and *2.6.*). The results are discussed separately for the individual groups of peptides.

3.1. Binding isotherm of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

The dissociation constant (K_d) for [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH was obtained from five replicate binding isotherms and was estimated by *MINSQ* non-linear least squares regression to be 0.48±0.08nM. A representative isotherm is shown in Fig. *3.1.*. Scatchard analysis of the binding isotherms, as described in section *2.4.2.1.*, showed that there was only one population of receptors present (Fig. *3.2.*). The number of receptors per cell varied from 5,000 to 25,000.

3.2. Binding affinity and biological activity of α-MSH fragments

α-MSH fragments were tested to investigate the influence of the terminal ends (amino acids 1-4 and 10-13) of the peptide. The fragments described in this section are Ac-α-MSH$_{(2-13)}$-NH$_2$, Ac-α-MSH$_{(3-13)}$-NH$_2$, Ac-α-MSH$_{(4-13)}$-NH$_2$ and Ac-α-MSH$_{(5-13)}$-NH$_2$ as well as Ac-α-MSH$_{(1-10)}$-NH$_2$, Ac-α-MSH$_{(1-11)}$-NH$_2$ and Ac-α-MSH$_{(1-12)}$-NH$_2$.

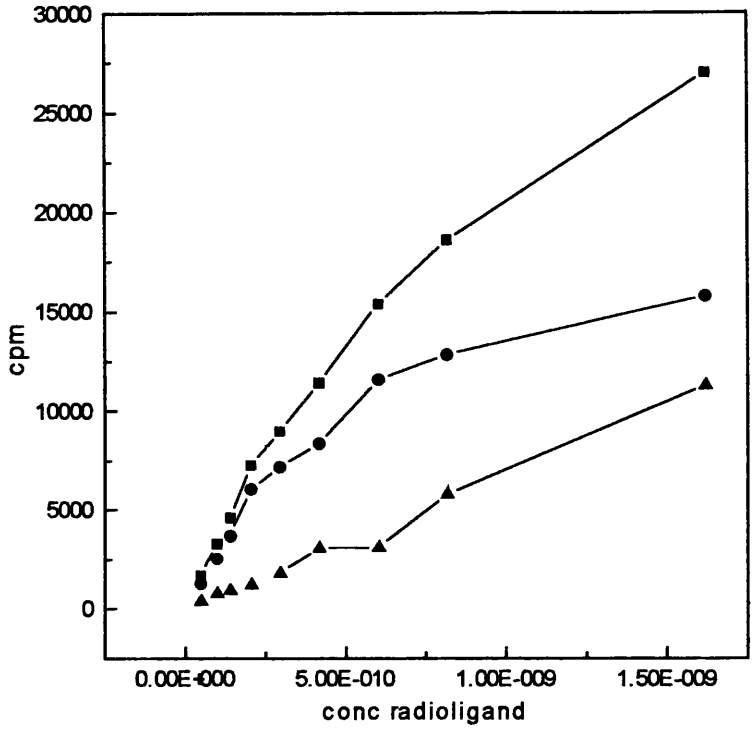


Fig. 3.1. Binding isotherm [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH to B16 murine melanoma cells at 0-4^{o}C. (■) total binding, (●) non-specific binding, (▲) specific binding.

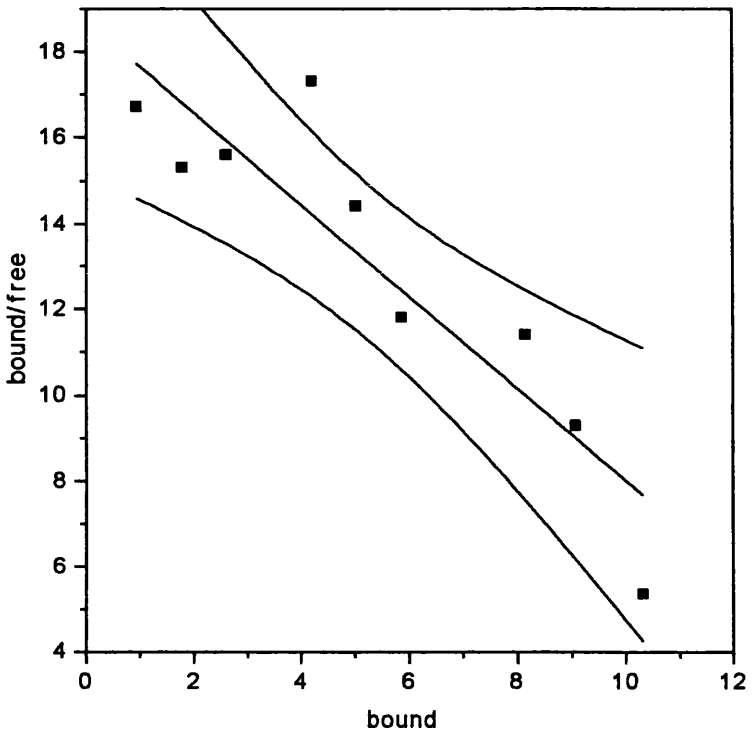


Fig. 3.2. Scatchard plot of a binding isotherm of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to B16 murine melanoma cells at 0-4^{o}C, curved line shows 95% confidence interval.

3.2.1. Receptor binding

Dissociation constants (Table *3.1.*) were estimated from the radioactivity associated with the cells as described in section *2.4.3.1.*. The means and standard deviations of three or more experiments were determined for each peptide. Values relative to α-MSH are given in Table *3.2.*. Representative examples of competitive binding isotherms are shown in Fig. *3.3.*. Loss of the N-terminal serine did not affect the affinity of the peptide, but removal of the serine-tyrosine dipeptide in positions 1 and 2 led to a peptide with 11 % of the affinity of α-MSH, thus being significantly different from both α-MSH and Ac-α-$MSH_{(2-13)}$-NH_2. Removal of the three N-terminal amino acids (Ac-α-$MSH_{(4-13)}$-NH_2) gave a peptide whose binding was not significantly different to α-MSH, Ac-α-$MSH_{(2-13)}$-NH_2 or Ac-α-$MSH_{(3-13)}$-NH_2. Removal of the four amino acids up to and including Met^4 reduced relative binding to 0.0016, and Ac-α-$MSH_{(5-13)}$-NH_2 had a significantly different binding affinity compared to α-MSH itself and the other N-terminal deletion fragments (Fig. *3.6.*).

Omission of the C-terminal amino acid valine did not significantly affect the dissociation constant, but additional loss of Pro^{12} gave a peptide whose binding was significantly lower than that of α-MSH itself (relative binding 0.05). Ac-α-$MSH_{(1-10)}$-NH_2 showed a significantly lower binding than the natural ligand (relative binding 0.0087), Ac-α-$MSH_{(1-12)}$-NH_2 and Ac-α-$MSH_{(1-11)}$-NH_2 ($p<0.05$). (Fig. *3.7.*)

3.2.2. Tyrosinase assay

EC_{50} values and standard deviations (Table *3.1.*) were obtained from the data as described in section *2.5.3.1.*. Each EC_{50} value is the mean of three or more replicate experiments. A representative example of the reproducibility is shown in Fig. *3.4.*.

Loss of the three N-terminal residues (Ac-α-$MSH_{(2-13)}$-NH_2, Ac-α-$MSH_{(3-13)}$-NH_2 and Ac-α-$MSH_{(4-13)}$-NH_2) did not significantly affect the biological activity of the peptide as measured by this assay. There was no significant difference in activity between any members of this group but removal of Met^4 in Ac-α-$MSH_{(5-13)}$-NH_2 produced a peptide which had significantly different tyrosinase activity to α-MSH itself (relative activity 0.001) and the other N-terminal deletion fragments (Fig. *3.6.*).

The activity of Ac-α-$MSH_{(1-12)}$-NH_2 was not significantly different to the native hormone but the activities of Ac-$MSH_{(1-11)}$-NH_2 and Ac-$MSH_{(1-10)}$-NH_2 were significantly lower, having relative potencies of 0.05 and 0.013 respectively. The activity of Ac-α-$MSH_{(1-11)}$-NH_2 was not significantly different to Ac-α-$MSH_{(1-12)}$-NH_2in contrast to the activity of Ac-α-$MSH_{(1-10)}$-NH_2 ($p<0.05$). The tyrosinase activities of Ac-α-$MSH_{(1-11)}$-NH_2 and Ac-α-$MSH_{(1-10)}$-NH_2 were not significantly different (Fig. *3.7.*).

3.2.3. Melanin assay

In replicate experiments, the cell number per well increased to 25,000 cells per well during the 72h period. An example of the dose-response curve is given in Fig. *3.5.*.

Data from this assay were analysed as described in section *2.6.1.1.* (Table *3.1.*, Fig.*3.6.*, Fig. *3.7.*). Values relative to α-MSH are given in Table *3.2.*. Results from the melanin assay were generally in good agreement with those from the tyrosinase assay with respect to the pattern of relative potencies with one exception in the C-terminal deletion sequences: Ac-α-$MSH_{(1-12)}$-NH_2 showed significantly different activity to α-MSH itself (relative potency 0.03) and Ac-$MSH_{(1-11)}$-NH_2 in the melanin assay, but not in the tyrosinase assay.

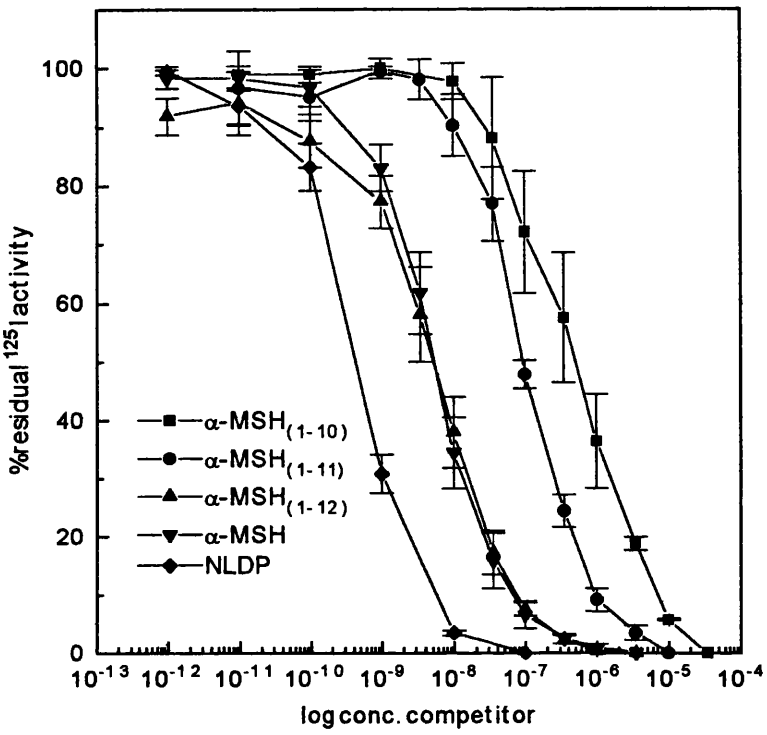


Fig. 3.3. Competition binding of C-terminal deletion fragments of α-MSH to B16 murine melanoma cells at 0-4°C.

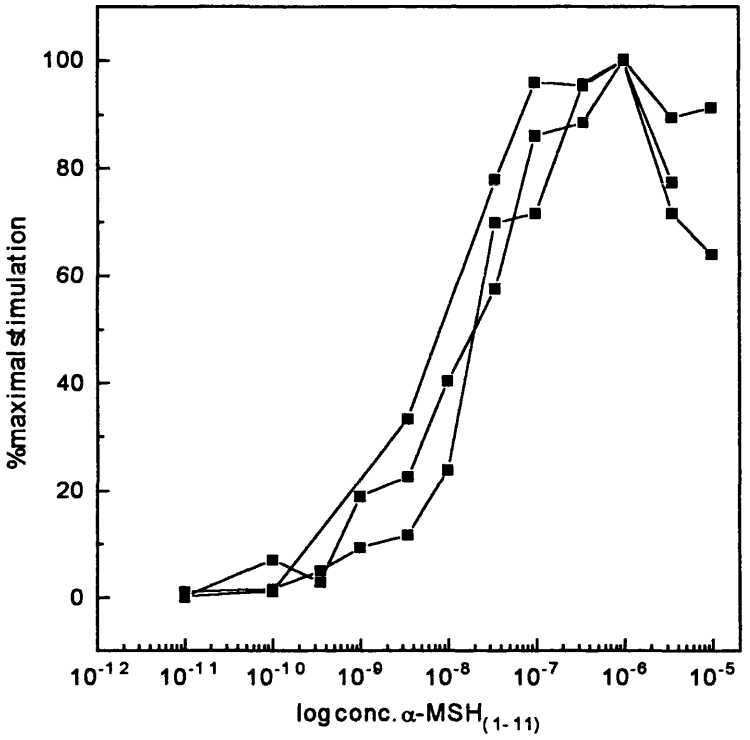


Fig. 3.4. Tyrosinase stimulation by α-$MSH_{(1-11)}$; replicate experiments.

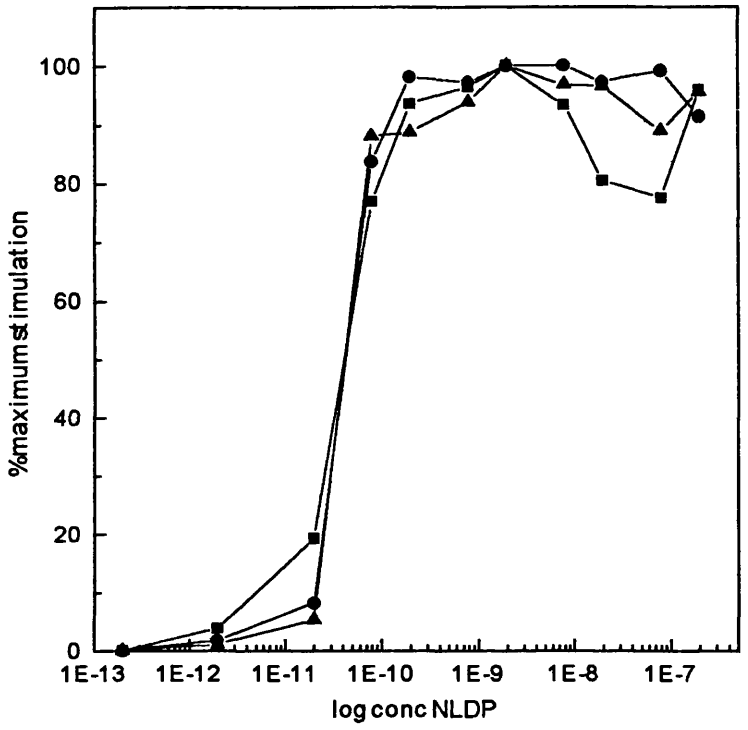


Fig. 3.5. Stimulation of melanin biosynthesis by [Nle^4,D-Phe^7]α-MSH (NLDP) as a function of its concentration in the culture medium

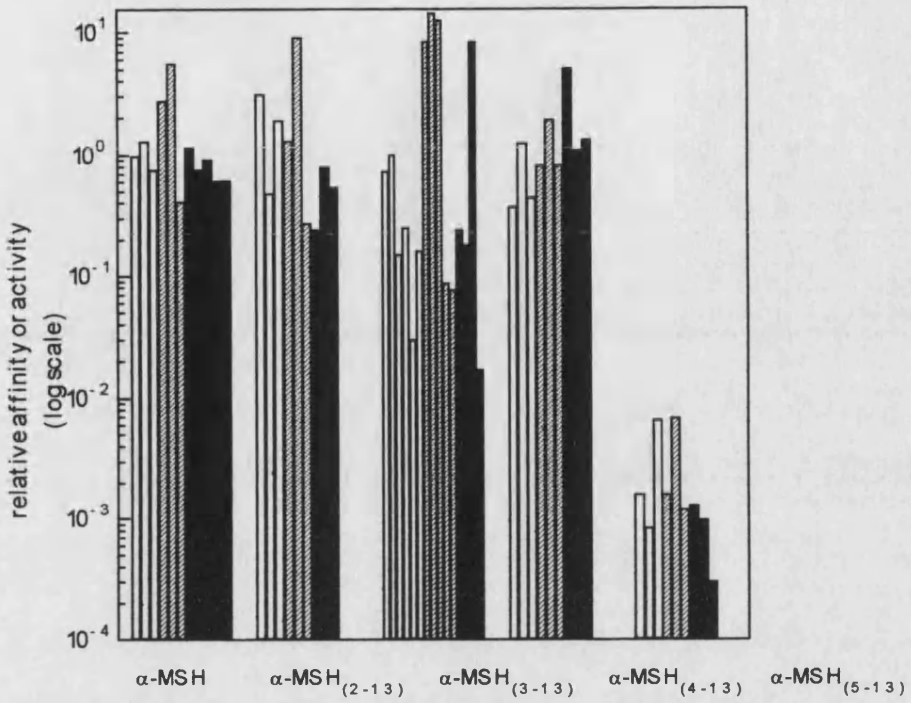


Fig. 3.6. Relative binding or activity (log scale) of α-MSH fragments lacking N-terminal amino acids. Individual bars represent single experiments: binding affinity, white; tyrosinase activity, cross-hatched; melanin biosynthesis, black

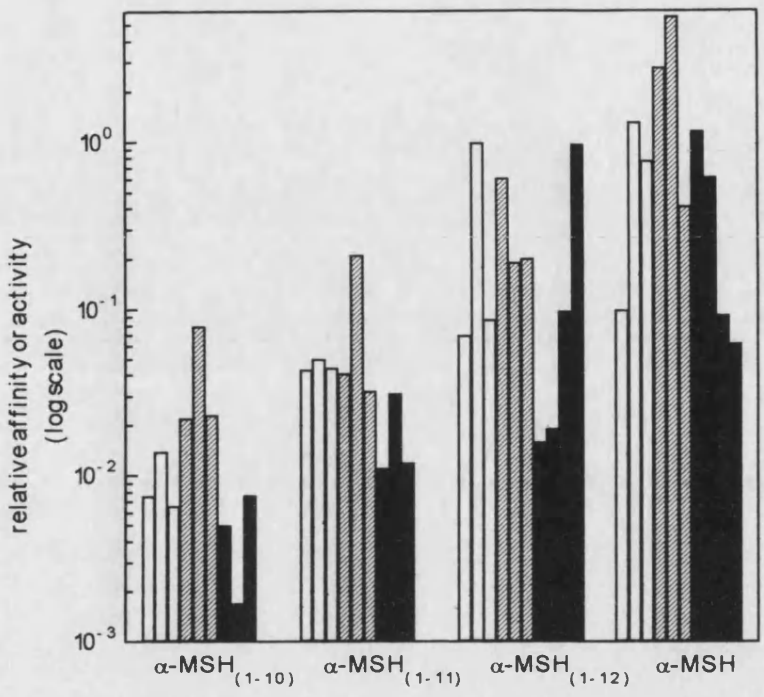


Fig. 3.7. Relative binding or activity (log scale) of α-MSH fragments lacking C-terminal amino acids. Individual bars represent single experiments: binding affinity, white; tyrosinase activity, cross-hatched; melanin biosynthesis, black

Table 3.1. Dissociation constants and EC_{50} values for α-MSH fragments. Each value is the mean of three or more experiments.

	Binding	Tyrosinase	Melanin
[Nle^4,D-Phe^7]α-MSH	2.02×10^{-9} $\pm7.81\times10^{-11}$ n=3	1.8×10^{-11} $\pm4.69\times10^{-12}$ n=4	2.18×10^{-10} $\pm2.50\times10^{-10}$ n=6
α-MSH	2.06×10^{-8} $\pm5.31\times10^{-9}$ n=3	6.1×10^{-10} $\pm7.73\times10^{-10}$ n=3	1.62×10^{-9} $\pm8.89\times10^{-10}$ n=5
Ac–α-MSH$_{(2-13)}$-NH_2	1.94×10^{-8} $\pm1.91\times10^{-8}$ n=3	9.46×10^{-10} $\pm1.19\times10^{-9}$ n=3	3.91×10^{-9} $\pm2.52\times10^{-9}$ n=3
Ac–α-MSH$_{(3-13)}$-NH_2	1.82×10^{-7} $\pm2.58\times10^{-7}$ n=6	3.01×10^{-9} $\pm4.06\times10^{-9}$ n=5	8.89×10^{-9} $\pm7.59\times10^{-9}$ n=4
Ac–α-MSH$_{(4-13)}$-NH_2	3.86×10^{-8} $\pm1.97\times10^{-8}$ n=3	6.70×10^{-10} $\pm1.39\times10^{-10}$ n=3	1.94×10^{-9} $\pm1.05\times10^{-9}$ n=3
Ac–α-MSH$_{(5-13)}$-NH_2	1.29×10^{-5} $\pm1.02\times10^{-5}$ n=3	5.87×10^{-7} $\pm2.61\times10^{-7}$ n=3	2.72×10^{-6} $\pm2.26\times10^{-6}$ n=3
Ac–α-MSH$_{(1-12)}$-NH_2	2.42×10^{-8} $\pm4.47\times10^{-9}$ n=3	1.94×10^{-9} $\pm1.20\times10^{-9}$ n=3	5.22×10^{-8} $\pm4.68\times10^{-8}$ n=4
Ac–α-MSH$_{(1-11)}$-NH_2	4.45×10^{-7} $\pm3.65\times10^{-8}$ n=3	1.23×10^{-8} $\pm8.38\times10^{-9}$ n=3	2.68×10^{-7} $\pm2.18\times10^{-7}$ n=3
Ac–α-MSH$_{(1-10)}$-NH_2	2.38×10^{-6} $\pm8.26\times10^{-7}$ n=3	4.42×10^{-8} $\pm2.84\times10^{-8}$ n=3	4.77×10^{-7} $\pm3.92\times10^{-7}$ n=3

Table 3.2. Affinity and activity constants of α-MSH fragments relative to α-MSH

	Binding	Tyrosinase	Melanin
[Nle^4,D-Phe^7]α-MSH	10.2	33.9	7.4
α-MSH	1.0	1.0	1.0
Ac–α-MSH$_{(2-13)}$-NH$_2$	1.06	0.64	0.41
Ac–α-MSH$_{(3-13)}$-NH$_2$	0.11	0.20	0.18
Ac–α-MSH$_{(4-13)}$-NH$_2$	0.53	0.91	0.84
Ac–α-MSH$_{(5-13)}$-NH$_2$	0.0016	0.001	0.0006
Ac–α-MSH$_{(1-12)}$-NH$_2$	0.85	0.31	0.03
Ac–α-MSH$_{(1-11)}$-NH$_2$	0.05	0.05	0.006
Ac–α-MSH$_{(1-10)}$-NH$_2$	0.0087	0.013	0.004

3.3. Binding affinity and biological activity of alanine analogues of α-MSH

This group of peptides contains thirteen analogues in which one amino acid has been replaced by alanine in order to study the influence of the amino acid side-chains and functional on receptor binding and biological activity.

3.3.1. Receptor Binding

Dissociation constants for the analogues were obtained as above (Table *3.3.*). The affinities relative to α-MSH are shown in Table *3.4.*. Alanine replacements in the N-terminal (amino acids 1-3) region did not significantly affect the affinity of the peptide. Within the 4-9 core of the peptide, alanine substitution of the amino acids Met^4, His^6, Phe^7, Arg^8 and Trp^9 lead to a significant drop in the binding to the receptor with the relative affinities of 0.0073, 0.012, 0.002, 0.00048 and 0.0005, respectively. Replacement of Glu^5 by alanine gave a peptide with an affinity that

was not significantly different from α-MSH. In the C-terminal part of the hormone (amino acids 10-13), replacement of Gly^{10}, Lys^{11} and Val^{13} gave peptides with affinities not significantly different from α-MSH whereas substitution of the Pro^{12} yielded a peptide with significantly lower affinity (9.6% of α-MSH) (Fig. *3.8.*).

3.3.2. Tyrosinase Assay

EC_{50} values are given in Table *3.3.*; data relative to α-MSH is shown in Table *3.4.*. Alanine substitution in either terminal region of the hormone (amino acids 1-3 or 10-13) did not significantly affect its biological activity. Within the peptide core, replacement of Glu^5, His^6 and Gly^{10} could be made without significant loss of activity. Alanine substitution of Met^4, Phe^7, Arg^8 and Trp^9 lead to significantly higher EC_{50} values and gave peptides with relative activities of 0.017, 0.0038, 0.01 and 0.008 respectively (Fig. *3.8.*).

Table 3.3. K_d and EC_{50} values for alanine analogues of α-MSH. Each value is the mean of three or more replicate experiments.

	Binding assay (K_d)	Tyrosinase assay (EC_{50})
[Nle^4,D-Phe^7]α-MSH	2.02×10^{-9} $\pm7.81\times10^{-11}$ n=3	1.80×10^{-11} $\pm4.69\times10^{-12}$ n=4
α-MSH	2.06×10^{-8} $\pm5.31\times10^{-9}$ n=3	6.1×10^{-10} $\pm7.73\times10^{-10}$ n=3
[Ala^1]α-MSH	5.09×10^{-8} $\pm2.94\times10^{-8}$ n=3	1.93×10^{-10} $\pm1.58\times10^{-10}$ n=3
[Ala^2]α-MSH	5.14×10^{-8} $\pm7.97\times10^{-9}$ n=3	5.59×10^{-10} $\pm3.98\times10^{-10}$ n=3
[Ala^3]α-MSH	1.72×10^{-8} $\pm6.52\times10^{-9}$ n=3	3.01×10^{-10} $\pm2.53\times10^{-10}$ n=4
[Ala^4]α-MSH	2.83×10^{-6} $\pm1.36\times10^{-6}$ n=3	3.69×10^{-8} $\pm2.34\times10^{-8}$ n=3

	Binding assay K_d	Tyrosinase assay EC_{50}
[Ala^5]α-MSH	7.2×10^{-9} $\pm 4.19 \times 10^{-9}$ n=5	8.70×10^{-10} $\pm 6.21 \times 10^{-10}$ n=4
[Ala^6]α-MSH	1.70×10^{-6} $\pm 1.20 \times 10^{-6}$ n=3	3.56×10^{-9} $\pm 1.29 \times 10^{-9}$ n=3
[Ala^7]α-MSH	1.05×10^{-5} $\pm 2.84 \times 10^{-6}$ n=4	1.09×10^{-7} $\pm 1.23 \times 10^{-7}$ n=4
[Ala^8]α-MSH	4.14×10^{-5} $\pm 5.89 \times 10^{-5}$ n=4	6.02×10^{-8} $\pm 4.02 \times 10^{-8}$ n=4
[Ala^9]α-MSH	4.12×10^{-5} $\pm 2.97 \times 10^{-5}$ n=3	7.56×10^{-8} $\pm 4.00 \times 10^{-8}$ n=3
[Ala^{10}]α-MSH	8.39×10^{-8} $\pm 1.43 \times 10^{-8}$ n=4	4.08×10^{-10} $\pm 2.64 \times 10^{-10}$ n=3
[Ala^{11}]α-MSH	3.61×10^{-8} $\pm 1.32 \times 10^{-8}$ n=3	2.35×10^{-9} $\pm 3.90 \times 10^{-9}$ n=4
[Ala^{12}]α-MSH	2.14×10^{-7} $\pm 7.12 \times 10^{-8}$ n=3	2.60×10^{-9} $\pm 2.51 \times 10^{-9}$ n=4
[Ala^{13}]α-MSH	1.81×10^{-8} $\pm 6.62 \times 10^{-9}$ n=3	4.87×10^{-10} $\pm 6.55 \times 10^{-10}$ n=5

Fig. 3.8. Relative affinity or activity of alanine-substituted analogues of α-MSH (log scale). Individual bars represent single experiments: cross-hatched, affinity; black, tyrosinase activity.

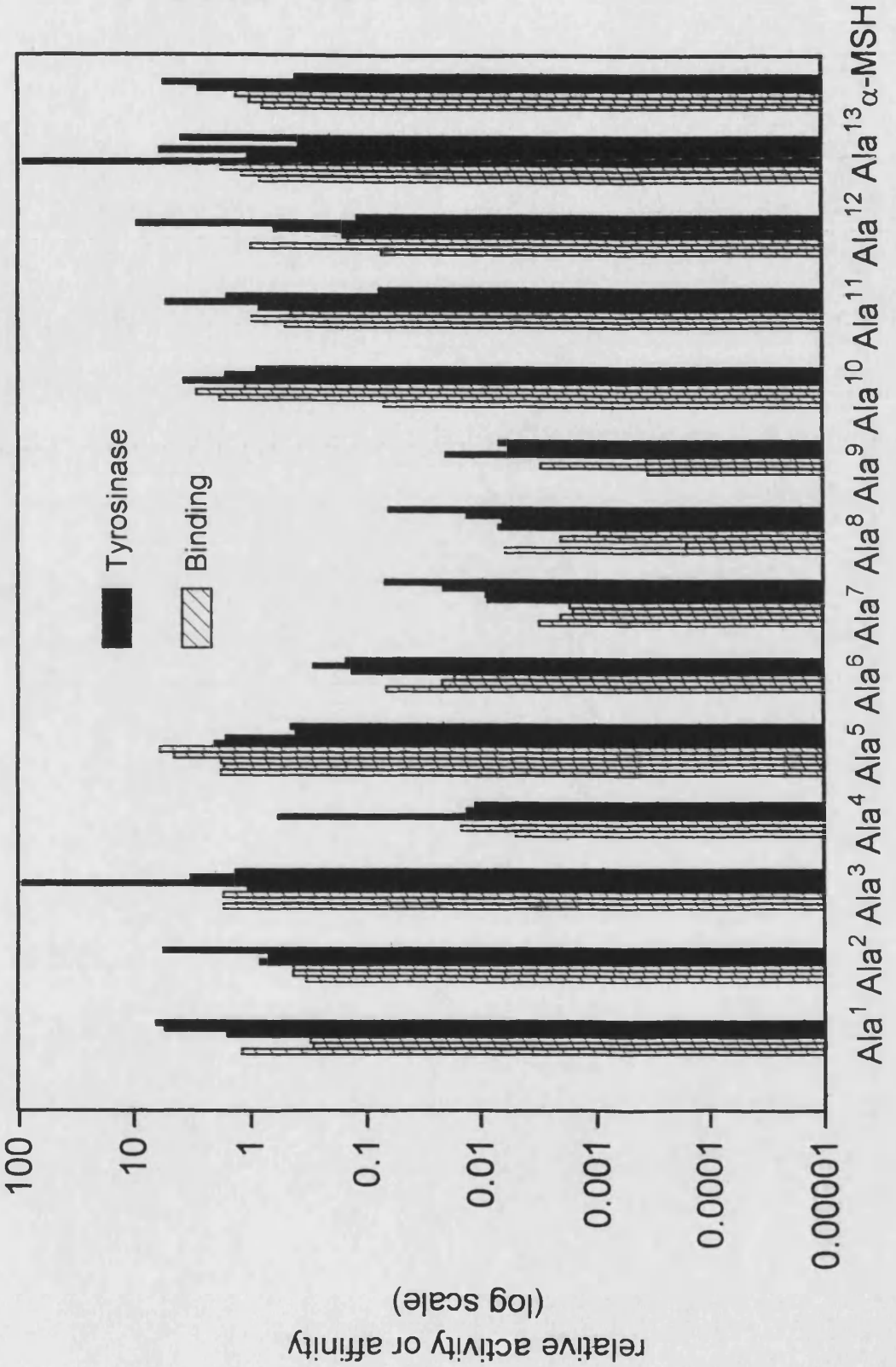


Table 3.4. Relative affinity and activity constants for alanine analogues of α-MSH.

	Binding	Tyrosinase
[Nle^4,D-Phe^7]α-MSH	10.2	33.9
α-MSH	1.0	1.0
[Ala^1]α-MSH	0.41	3.16
[Ala^2]α-MSH	0.40	1.09
[Ala^3]α-MSH	1.20	2.03
[Ala^4]α-MSH	0.0073	0.017
[Ala^5]α-MSH	2.86	0.70
[Ala^6]α-MSH	0.012	0.17
[Ala^7]α-MSH	0.002	0.0038
[Ala^8]α-MSH	0.00048	0.010
[Ala^9]α-MSH	0.0005	0.008
[Ala^{10}]α-MSH	0.25	1.49
[Ala^{11}]α-MSH	0.57	0.25
[Ala^{12}]α-MSH	0.096	0.23
[Ala^{13}]α-MSH	1.14	1.25

3.4. D-Phe^7 analogues of α-MSH

D-amino acid analogues of α-MSH, in particular D-Phe^7 analogues have been studied following observations that the bioactivity of α-MSH is increased when racemisation occurs. [Nle^4,D-Phe^7]α-MSH has been reported to be a prolonged

acting, 'superpotent' analogue of the hormone (Sawyer *et al.*, 1982). Due to its greater stability and ease of radioiodination it is now used as a standard comparison throughout the literature.

3.4.1. Receptor binding

Estimates of the dissociation constants are shown in Table *3.5.*; the relative affinity is given in Table *3.6.*. The binding affinity of [Nle^4,D-Phe^7]α-MSH is 10 times higher than that of α-MSH. The receptor binding of [Nle^4,Asp^5,D-Phe^7,Lys^{10},Arg^{11}]α-MSH and [Nle^4,D-Phe^7,D-Trp^9]α-MSH is in the same range, being significantly higher than α-MSH (6.38 and 7.98, respectively), but not significantly different from [Nle^4,D-Phe^7]α-MSH (Fig. *3.9.*).

3.4.2. Tyrosinase assay

Both [Nle^4,D-Phe^7]α-MSH and [Nle^4,Asp^5,D-Phe^7,Lys^{10},Arg^{11}]α-MSH showed a significantly higher activity than α-MSH with their respective EC_{50} values being 33.1 and 39.3 times higher than of the native hormone. [Nle^4,D-Phe^7,D-Trp^9]α-MSH was significantly different from α-MSH ($p<0.05$) with 12.2 times its activity (Fig. *3.9.*).

3.4.3. Melanin assay

[Nle^4,D-Phe^7]α-MSH and [Nle^4,Asp^5,D-Phe^7,Lys^{10},Arg^{11}]α-MSH were significantly more active than α-MSH in this assay. [Nle^4,D-Phe^7,D-Trp^9]α-MSH was not tested in this assay.

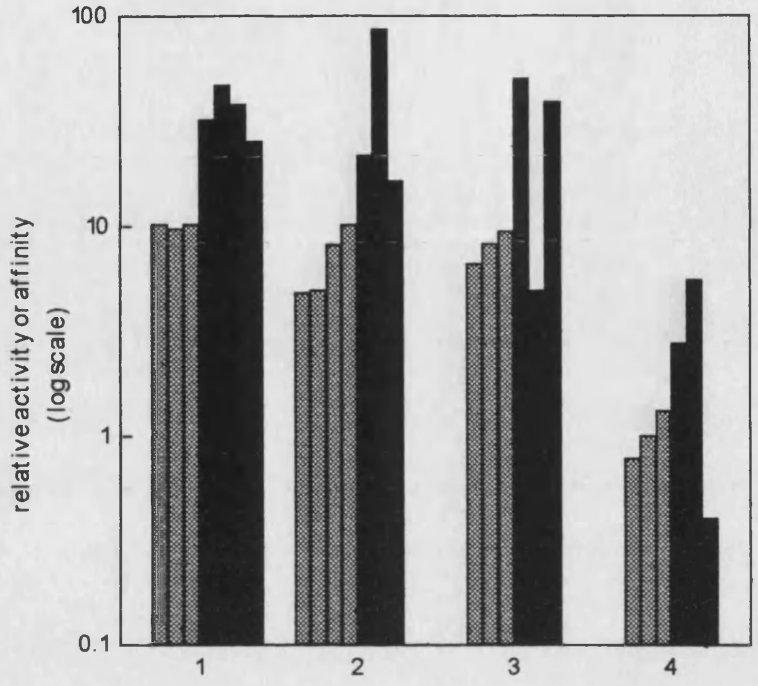


Fig. 3.9. Relative affinity or activity of D-Phe^7 analogues of α-MSH (log scale). Individual bars represent single experiments: cross-hatched, affinity; black, tyrosinase activity. (1) = [Nle^4,D-Phe^7]α-MSH, (2) = [Nle^4,Asp^5,D-Phe^7,Lys^{10}, Arg^{11}]α-MSH, (3) = [Nle^4,D-Phe^{7}D-Trp^9]α-MSH, (4) = α-MSH.

Table 3.5. Dissociation constants and EC_{50} values of D-Phe^7 analogues of α-MSH. Each value is the mean of three or more experiments.

	Binding K_d	Tyrosinase EC_{50}	Melanin EC_{50}
[Nle^4,D-Phe^7]α-MSH	2.02x 10^{-9} ±7.81x 10^{-11} n=3	1.80x 10^{-11} ±4.69x 10^{-12} n=4	2.18x 10^{-10} ±2.50x 10^{-10} n=6
[Nle^4,Asp^5,D-Phe^7, Lys^{10},Arg^{11}]α-MSH	3.23x 10^{-9} ±1.14x 10^{-10} n=4	1.56x 10^{-11} ±1.87x 10^{-11} n=3	1.61x 10^{-10} ±1.90x 10^{-10} n=3
[Nle^4,D-Phe^7,D-Trp^9]α-MSH	2.58x 10^{-9} ±4.72x 10^{-10} n=3	5.02x 10^{-11} ±6.30x 10^{-11} n=3	n.d.

Table 3.6. Affinity and biological activity relative to α-MSH

	Binding	Tyrosinase	Melanin
[Nle^4,D-Phe^7]α-MSH	10.2	33.9	7.4
[Nle^4,Asp^5,D-Phe^7 Lys^{10},Arg^{11}]α-MSH	6.38	39.1	10.1
[Nle^4,D-Phe^7,D-Trp^9]α-MSH	7.98	12.2	n.d.

3.5. Cyclic analogues of α-MSH

Two cyclic α-MSH derivatives containing a disulfide bridge between positions 4 and 10 have been assayed for receptor binding and biological activity. Also, the linear analogue [Cys^4,Cys^{10}]α-MSH is included in this section to allow a comparison of the relative affinities and activities of the analogues with a molecule of the same structure. It was also attempted to produce a cyclic analogue of [Nle^4,Asp^5,D-Phe^7,Lys^{10},Arg^{11}]α-MSH with a lactam ring between positions 5 and 10, but as the cyclisation failed, the data of the linear analogue are described in section *3.4.*.

3.5.1. Competitive Binding

The dissociation constants are given in Table *3.7.*; data relative to α-MSH are shown in Table *3.8.*. The affinity of [Cys^4,Cys^{10}]α-MSH (0.06%) and [$\overline{\text{Cys}^4,\text{Cys}^{10}}$]α-MSH (0.24%) were both significantly different from α-MSH, but they were also significantly different from each other. The binding of Ac-[$\overline{\text{Cys}^4}$,D-$\overline{\text{Phe}^7,\text{Cys}^{10}}$]α-MSH$_{(4\text{-}13)}$-NH$_2$ was three times higher than that of the natural ligand and thus significantly different from α-MSH and the other two peptides in this group (Fig. *3.10.*).

3.5.2. Tyrosinase Assay

The EC_{50} values are shown in Table *3.7.* and the activity relative to α-MSH in Table *3.8.*. The tyrosinase stimulating activity of [Cys^4,Cys^{10}]α-MSH and [$\overline{\text{Cys}^4,\text{Cys}^{10}}$]α-MSH was not significantly different from that of α-MSH, whereas they were different from Ac-[Cys^4,Cys^{10}]α-MSH$_{(4\text{-}13)}$-NH$_2$. The biological activity of Ac-[$\overline{\text{Cys}^4,\text{D-Phe}^7,\text{Cys}^{10}}$]α-MSH$_{(4\text{-}13)}$-NH$_2$ was significantly different from α-MSH with $p<0.05$ (Fig. *3.10.*).

3.5.3. Melanin Assay

Activity data are shown in Tables *3.5.1.* and *3.5.2.*. The biological activity of [Cys^4,Cys^{10}]α-MSH and [$\overline{\text{Cys}^4,\text{Cys}^{10}}$]α-MSH was not significantly different from α-MSH in this assay; Ac-[$\overline{\text{Cys}^4,\text{D-Phe}^7,\text{Cys}^{10}}$]α-MSH$_{(4\text{-}13)}$-NH$_2$ had not been tested.

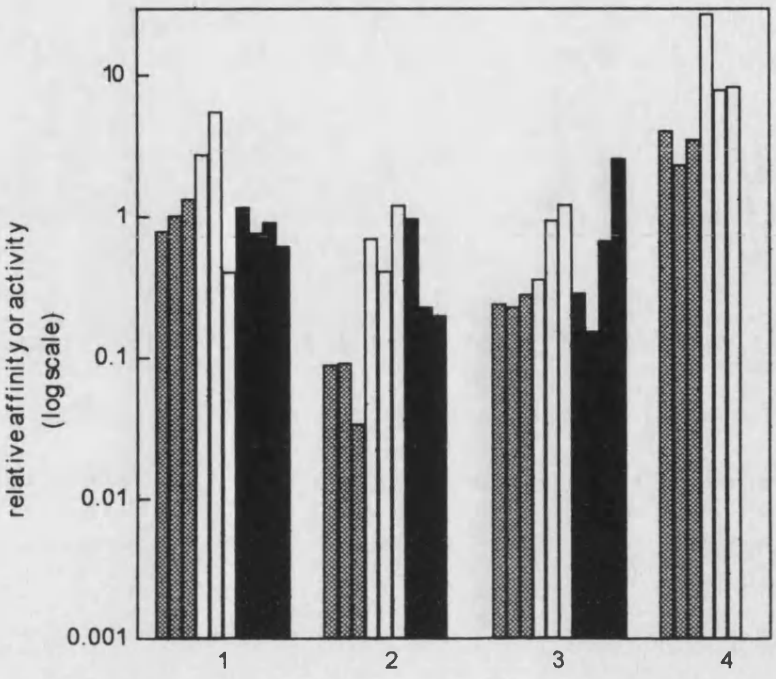


Fig. 3.10. Relative affinity or activity of linear and cyclic analogues of [Cys^4,Cys^{10}]α-MSH (log scale). Individual bars represent single experiments: cross-hatched, binding affinity; white, tyrosinase stimulation; black, melanin biosynthesis. (1) = α-MSH, (2) = [Cys^4,Cys^{10}]α-MSH, (3) = [$\overline{\text{Cys}^4,\text{Cys}^{10}}$]α-MSH, (4) = [$\overline{\text{Cys}^4,\text{D-Phe}^7,\text{Cys}^{10}}$]α-MSH$_{(4\text{-}13)}$.

Table 3.7. Dissociation constants and EC_{50}s of cyclic α-MSH analogues. Each value is the mean of three or more experiments.

	Binding affinity K_d	Tyrosinase assay EC_{50}	Melanin assay EC_{50}
[Cys^4,Cys^{10}]α-MSH	3.52×10^{-7} $\pm2.17\times10^{-7}$ n=3	9.60×10^{-10} $\pm5.02\times10^{-10}$ n=3	5.61×10^{-9} $\pm3.46\times10^{-9}$ n=3
[$\overline{\text{Cys}^4,\text{Cys}^{10}}$]α-MSH	8.32×10^{-8} $\pm8.99\times10^{-9}$ n=3	9.51×10^{-10} $\pm6.53\times10^{-10}$ n=3	4.83×10^{-9} $\pm4.38\times10^{-9}$ n=4
Ac-[$\overline{\text{Cys}^4,\text{D-Phe}^7,\text{Cys}^{10}}$] α-MSH$_{(4-13)}$-NH$_2$	6.66×10^{-9} $\pm2.02\times10^{-9}$ n=3	5.80×10^{-11} $\pm3.09\times10^{-11}$ n=3	n.d.

Table 3.8. Relative affinities and activities of cyclic α-MSH analogues

	Binding affinity	Tyrosinase assay	Melanin assay
[Cys^4,Cys^{10}]α-MSH	0.06	0.63	0.28
[$\overline{\text{Cys}^4,\text{Cys}^{10}}$]α-MSH	0.24	0.64	0.33
Ac-[$\overline{\text{Cys}^4}$,D-Phe^7, $\overline{\text{Cys}^{10}}$]α-MSH$_{(4-13)}$-NH$_2$	3.09	10.5	n.d.

3.6. Other MSH peptides

Both desacetyl-α-MSH and the free acid have been tested, the desacetyl peptide, which occurs naturally in dogfish, mainly for comparison with the MC3 receptor. γ_1-MSH has not been tested for binding to this receptor before; other γ-MSH peptides have not been tested as they are expected to exhibit similar or lower affinity due to their extended C-terminus which might not be recognised by the receptor.

3.6.1. Competitive Binding/Tyrosinase Assay

There was no significant difference in binding or tyrosinase stimulating activity compared to α-MSH. The dissociation constant was estimated to be $1.68\pm0.598\times10^{-8}$M (n=3). The EC50 was determined as $7.18\pm5.19\times10^{-10}$M (n=3). Desacetyl-α-MSH and γ_1-MSH have only been tested in the binding assay. Their dissociation constants were found to be 15.0nM (±15.2nM; n=3) and 3.79μM (± 2.02μM; n=3), respectively.

CHAPTER 4

Discussion

Binding Affinity and Biological Activity of α-MSH Derivatives in B16 Mouse Melanoma Cells

Although a number of the peptides including the terminal deletion fragments and the analogues of [Cys^4,Cys^{10}]α-MSH described here have already been tested for their affinity or activity in various reptilian and mammalian bioassays (for a review see Eberle, 1988), their activities have not been systematically studied in relation to their receptor binding affinity. Also, numerous single and multiple amino acid replacements have been made within the sequence, but a systematic investigation into the role of individual amino acid side-chains in receptor-ligand interaction has not yet been made. Direct comparison of binding affinity and biological activity allows an estimate of the intrinsic efficacy of the analogues. Tyrosinase activity and melanin biosynthesis assays have been carried out in parallel for some of the peptides tested. Results from both assays were generally in good agreement with each other, but because of the greater experimental error associated with the melanin biosynthesis assay, it was thought that, although more convenient, it is less valuable than the tyrosinase assay in determining the biological response of the cells.

4.1. Binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

Binding isotherms with the mono-iodinated α-MSH derivative [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH showed that this ligand bound specifically to the cell surface receptor (Fig. *3.2.* and *3.3.*). The dissociation constant was estimated to be

0.48nM±0.08nM. This is in agreement with values previously obtained with the same assay in our laboratory (Erskine-Grout, 1993). The population of receptors is believed to be homogeneous as shown by Scatchard analysis (Scatchard, 1949). Their number varied from 5,000 to 20,000, but the variable number of binding sites did not affect the binding affinities of the ligands.

α-MSH bound to the receptor with an affinity of 20.6±5.31nM. This is a slightly lower affinity than reported by Eberle (1988), who reported a value of 1.3nM for α-MSH. This disagreement might be due to different experimental conditions used (incubation for 3h at 15°C, use of MEM medium). It is not likely to be caused by the use of a different strain of B16 cells as they would be expected to express a very similar receptor, although there are some reports of heterogeneity of the α-MSH receptor among B16 murine melanoma cell subclones (Solca *et al.*, 1991).

4.2. Influence of terminal amino acids

Since the structure-activity relationships of α-MSH were first studied and it was found that the pentapeptide sequence $\alpha\text{-MSH}_{(6\text{-}10)}$ displayed weak but distinct pigment-dispersing activity (Schwyzer and Li, 1958), it has been known that neither of the termini of the peptide are crucial for the peptide's biological action. The unimportance of the N-terminus has been established in α-MSH fragments (see Eberle, 1988; p336) and cyclic analogues (Knittel *et al.*, 1983); data from Eberle comparing several assays, however, suggest that the terminal amino acids are more influencial in the *Rana pipiens* melanophore assay than in any other assay. The C-terminal amino acids 10-13 are clearly more important than the N-terminal residues even though biological activity is retained when they are omitted. There is some evidence that the C-terminal tripeptide $\alpha\text{-MSH}_{(10\text{-}13)}$ contains a "secondary message sequence" as it was shown to display pigment dispersing activity (Eberle and Schwyzer, 1975) as well as some of the CNS activities of α-

MSH, such as anti-inflammatory action (Hiltz *et al.*, 1991). Other studies, however, indicate that the presence of the tripeptide does not significantly increase the biological activity of some α-MSH fragments (Hruby *et al.*, 1987; Sawyer *et al.*, 1991). Eberle suggested that as α-$MSH_{(9-13)}$ exhibited about the same activity as the central hexapeptide α-$MSH_{(5-10)}$, but the dipeptides Ac-Trp-Gly-OH and Ac-Pro-Val-OH were inactive, Lys^{11} might play a key role in the biological activity of this C-terminal α-MSH fragment. Studies on the influence of C-terminal amino acids on the activity of fragments of [$\overline{Cys^4,Cys^{10}}$]α-MSH have shown that while Val^{13} did not contribute to the biological activity of the peptide, loss of the proline resulted in a 10-100 fold drop in activity in three different assays, and subsequent omission of Lys^{11} reduced the activity a further 5-20 fold (Cody *et al.*, 1984).

In the work reported here, consecutive deletion of amino acids 1-3 from the N-terminus of α-MSH gave three peptides whose biological activity was not significantly different from α-MSH or from each other, but the binding affinity of Ac-α-$MSH_{(3-13)}$-NH_2 was significantly different from both α-MSH and Ac-α-$MSH_{(2-13)}$-NH_2, but not from Ac-α-$MSH_{(4-13)}$-NH_2. As Ac-α-$MSH_{(4-13)}$-NH_2 showed similar biological action to α-MSH, the N-terminus is obviously not required for either receptor binding or biological activity (Fig. *3.6.*, *3.7.*). The results obtained with Ac-α-$MSH_{(2-13)}$-NH_2 and Ac-α-$MSH_{(3-13)}$-NH_2, however, suggest, that if the N-terminus is present, the tyrosine in position 2 has a role in interacting with the receptor, probably in a non-specific manner due to its relative hydrophobicity as compared to serine. This would also explain why the affinity of the analogue [Nle^4,D-Phe^7]α-MSH (K_d=2.02nM) is increased upon radioiodination ([^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH: K_d=0.48nM). These results are generally in agreement with results reported in the literature (see Eberle, 1988).

Ac-α-$MSH_{(1-12)}$-NH_2 did not have a significantly different binding affinity or tyrosinase stimulating activity from α-MSH, but values from the melanin assay

showed a statistically significant difference to the natural ligand. As this is the difference in biological activity assayed by tyrosinase or melanin assay, it is likely to be due to the experimental technique rather than to a genuine difference in activity. Ac-α-$MSH_{(1-11)}$-NH_2 showed only 5% of the binding and tyrosinase stimulating activity and 0.6% of the melanin biosynthesis compared to α-MSH. Finally, Ac-α-$MSH_{(1-10)}$-NH_2 with approximately 1% of the affinity and tyrosinase stimulating activity and 0.3% of the biological activity in the melanin biosynthesis assay, showed significantly different receptor binding from Ac-α-$MSH_{(1-11)}$-NH_2, but there was no significant difference in biological activity between the two fragments. These results indicate that the proline in position 12 might be the key residue in this terminal end of α-MSH, rather than the Lys^{11} as previously suggested (Eberle, 1988). The data is in accordance with findings obtained by Cody *et al.* (1984) for fragments of [$\overline{Cys^4,Cys^{10}}$]α-MSH.

4.3. Influence of single amino acid replacement

Single amino acid replacement by alanine is a technique that has previously been employed in the investigation of the ligand receptor interaction of physiological peptides (Beck-Sickinger *et al.*, 1990; O'Donnell *et al.*, 1991; Peeters *et al.*, 1992). It provides insight into the importance of individual amino acid side-chains for the interaction with the receptor while maintaining the stereochemistry of the peptide backbone; alanine is the shortest amino acid this can be obtained with. For α-MSH, the importance of the terminal amino acids on its affinity and activity has been widely studied (see Eberle, 1988 and section *4.2.*), but the importance of individual residues within the core sequence has not been investigated systematically using both activity and binding assays in a mammalian cell line.

Substitution of the N-terminal amino acids Ser^1, Tyr^2 and Ser^3 did not significantly affect the binding affinity or biological activity of α-MSH. This is in agreement

with observations made with the α-MSH fragments, Ac-α-$MSH_{(2-13)}$-NH_2, Ac-α-$MSH_{(3-13)}$-NH_2 and Ac-α-$MSH_{(4-13)}$-NH_2 (see section *4.1.*).

Within the peptide core, amino acids 4-9, alanine replacement generally decreased the affinity and/or activity of the hormone. Substitution of the glutamic acid residue in position 5, however, unexpectedly led to a peptide with similar biological properties to α-MSH. In spite of having a charged side-chain, this result suggests that it does not participate in the interaction with the receptor. Previous studies have shown that although replacement of Glu by Gln reduced the activity of α-MSH in the frog skin bioassay 20 times, this substitution was possible without further loss of activity in α-$MSH_{(5-10)}$ (Blake *et al.*, 1970). Attachment of large groups to the side-chain of Glu was also reported to reduce activity (Eberle, 1988). Studies by Al-Obeidi *et al.* (1989a,b) were carried out with linear and cyclic analogues of [Nle^4,D-Phe^7]α-MSH, Ac-[Nle^4,Glu^5,D-Phe^7,Lys^{10},Gly^{11}]α-$MSH_{(4-13)}$-NH_2 and Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}, Gly^{11}]α-$MSH_{(4-13)}$-NH_2, and showed that lactam cyclisation of these peptides through the side-chains of Glu^5 or Asp^5 and Lys^{10} is possible without loss of activity in the frog skin assay and resulted in even higher activities in the lizard skin assay. Hence, amidation and conformational restraint at residue 5 does not affect the biological response of the hormone.

[Ala^4]α-MSH showed significantly reduced binding and biological activity compared to α-MSH. Methionine could however be replaced isosterically by norleucine (Sawyer *et al.*, 1982; Siegrist *et al.*, 1988) without loss of affinity or activity. Substitution of methionine with cysteine in linear and cyclic [Cys^4,Cys^{10}]α-MSH derivatives is also possible without affecting the properties of the peptide (Sawyer *et al.*, 1980; Eberle, 1988; section *3.5.*). Oxidation of the residue to Met(O) or Met(O_2) leads to almost complete loss of binding affinity and biological activity (Eberle *et al.*, 1978; Siegrist *et al.*, 1988). Introduction of a tyrosine residue at this position in the α-$MSH_{(4-10)}$ and α-$MSH_{(4-11)}$ analogues gave peptides with significantly less activity in both the frog and the lizard skin bioassay,

which were only partial agonists in the Cloudman S91 adenylate cyclase assay (Wilkes *et al.*, 1984).

Introduction of an alanine residue in position 6 decreased the binding affinity significantly to 0.012 times that of α-MSH whereas there was no difference in the biological activity (0.17) compared to the native ligand. This suggest that full receptor binding might not be required to trigger the biological response.

Alanine substitution in positions 7, 8 and 9 clearly affected the binding affinity and biological activity the most. While replacement of Phe^7 by alanine affected binding and activity to the same extent (relative values being 0.002 and 0.0038), exchange of Arg^8 and Trp^9 seemed to reduce the binding affinity more than the biological activity (0.00048 vs. 0.01 and 0.0005 vs. 0.008, respectively). Previous studies have shown that replacement of Phe^7 by its D-isomer is possible while any other substitution in position 7 and 8 significantly decreases binding and activity of the hormone (Eberle, 1988). Introduction of a D-Trp^9 is also possible without affecting the peptide (section *3.4.*). Trp^9 could also be replaced by pentaphenylalanine (van Nispen *et al.*, 1970) and leucine (Eberle and Schwyzer, 1979) and retain a relatively high amount of activity in the frog skin assay. Attachment of a 2-nitro-4-azidophenylsulphenyl (Naps) photoaffinity label to the tryptophan led to a peptide with slightly higher activity than α-MSH (Scimonelli and Eberle, 1987). It seems that this amino acid can generally be replaced by other aromatic residues, or non-aromatic amino acids of a certain size and lipophilicity. Any substitution is likely to affect affinity more than activity.

In the C-terminal part of the peptide, substitution of the glycine in position 10 did not affect the biological properties of the peptide. This might be due to the structural similarity of the two amino acids, although alanine is expected to restrict the conformational freedom of the peptide backbone. Previously, it has been

suggested that glycine acts as a spacer rather than being an important residue in itself (Medzihradsky, 1976). Introduction of other residues such as cysteine in [Cys^4,Cys^{10}]α-MSH (Sawyer *et al.*, 1980; Eberle, 1988) or lysine in Ac-[Nle^4,Glu^5,D-Phe^7,Lys^{10},Gly^{11}]α-MSH$_{(4\text{-}13)}$-NH$_2$ and Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}, Gly^{11}]α-MSH$_{(4\text{-}13)}$-NH$_2$ (Al-Obeidi *et al.*, 1989a,b) appears to be possible in both linear and cyclic analogues without major effects on the peptide's action.

[Ala^{11}]α-MSH had approximately the same affinity and activity as the native hormone. This is in contradiction to earlier results which suggested that the basic side-chain of Lys^{11} might be important for the interaction with the receptor, and replacement with serine or glutamic acid decreased the biological activity by a factor of 20-40 (Eberle, 1988). It was shown in our laboratory that it is possible to attach a photoaffinity label, 4-(1-azi-2,2,2-trifluoroethyl)benzoic acid (ATB), onto the lysine without significant loss of affinity (Ahmed *et al.*, 1992). Replacement by arginine (section *3.4.*) and glycine (Al-Obeidi *et al.*, 1989a,b) is also possible without affecting the biological properties of the molecule.

Substitution of the proline in position 12 with D-proline, norvaline or glycine has been reported to significantly decrease the biological activity of α-MSH in several reptilian bioassays and the Cloudman S91 tyrosinase assay (Eberle, 1988). Replacement by alanine reduced the binding affinity to 10% in B16 melanoma cells while the biological activity was not significantly lower than that of the native hormone.

Substitution of Val^{13} was possible without affecting the affinity and activity of the peptide; earlier findings suggested that this residue can be replaced as long as it remains lipophilic (Eberle, 1988).

4.4. Cyclic α-MSH analogues

Cyclic, conformationally constrained peptide derivatives are valuable tools in the study of structure-conformation relationships. They adopt a more defined range of conformation than linear peptides and are thus more amenable to conformational analysis by NMR spectroscopy, circular dichroism or IR spectroscopy (Hadley, 1988). Also, they possess an energetic advantage compared to linear molecules as they do not have to adopt a biologically active conformation prior to receptor binding. Two ways of introducing a ring-structure into a peptide molecule have been attempted for α-MSH: formation of a disulfide bridge and lactam cyclisation. Both these attempts have been made for α-MSH. The first cyclic α-MSH analogue to be described was [$\overline{Cys^4,Cys^{10}}$]α-MSH (Sawyer *et al.*, 1982), which was originally reported to be 10,000 times as active as α-MSH in the frog skin bioassay. Later, this value had to be revised and it was found to exhibit a 10 fold higher activity in this assay (Eberle, 1988). The reason this particular structure was chosen was that it seemed to stabilise a reverse turn of the peptide backbone between positions 6 and 9, a conformation that had been proposed after the discovery of [Nle^4,D-Phe^7]α-MSH as a superpotent α-MSH analogue (Sawyer *et al.*, 1980). The D-Phe^7 replacement appeared to favour the β-turn conformation in this part of the active side of the peptide. In [$\overline{Cys^4,Cys^{10}}$]α-MSH, both the putative active sites, residues 6-9 and 11-13, were left unchanged, and the amino acid substitutions made in position 4 and 10 can be considered pseudoisosteric. A number of other substitutes for position 4 were tested in cyclic compounds (Lebl *et al.*, 1984a,b), and while replacement of Cys with D-Cys or 3-mercapto propionic acid is possible without significant loss of activity, introduction of mercapto acetic acid reduces the biological activity of the cyclic compound dramatically. This is believed to be due to the reduced ring size in this molecule compared to [$\overline{Cys^4,Cys^{10}}$]α-MSH. Influence of the C-terminal amino acids (11-13) was then investigated and it was found, that Val ^{13} is not required, but there is a drastic loss

of activity following the loss of Pro^{12}, with a less dramatic loss after subsequent omission of Lys^{11} (Cody *et al.*, 1984). This is in agreement with our findings for linear α-MSH fragments (see section *4.1.*). Nearly the same pattern can be observed in cyclic fragments containing the D-Phe^7 residue, although omission of Pro^{12} and Lys^{11} in the D-Phe^7 form clearly affected the peptide less than in the L-Phe^7 analogue (Cody *et al.*, 1988).

No receptor binding data has been reported for any of the cyclic compounds so far, nor have they been compared to their linear analogues. In the B16 murine melanoma cells, both linear and cyclic [Cys^4,Cys^{10}]α-MSH showed a significantly lower affinity for the receptor (see section *3.5.*); their affinities were also significantly different from each other with the linear analogue possessing only 23% of the binding affinity of the cyclic peptide. In the tyrosinase and melanin assay, however, there was no difference between the two forms. This indicates that while the cyclic form is stable at 0-4°C, the disulfide bridge might be degraded at 37°C giving the linear peptide. A D-Phe^7 analogue, Ac-[$\overline{Cys^4,D\text{-}Phe^7,Cys^{10}}$]α-$MSH_{(4\text{-}13)}$-$NH_2$ was also tested and it showed approximately the same biological properties as [Nle^4,D-Phe^7]α-MSH with its relative affinity and tyrosinase stimulating activity being 3.09 and 10.5, respectively, compared to α-MSH. The increase in activity observed here is interesting considering that the cyclisation itself is said to stabilise the reverse turn conformation of the peptide backbone (Sawyer *et al.*, 1982); possibly the D-amino acid further stabilises any existing structures, or the molecule was actually present in an opened form, which then profits from the introduction of the D-Phe residue. The observation is in agreement with literature values for the bioactivity of D-Phe substituted analogues of [$\overline{Cys^4,Cys^{10}}$]α-MSH (Cody *et al.*, 1988).

Cyclic lactam analogues of α-MSH have been made in two different positions. The first cyclic lactam compounds were Ac-[Nle^4,D-$\overline{Orn^5,Glu^8}$]α-$MSH_{(4\text{-}11)}$-NH_2 and

its D-Phe^7 analogue, giving a 17-membered ring (Sugg *et al.*, 1988). The choice of this particular ring structure was based on predictions by Nikiforovich *et al.* (1981) that the side-chains in positions 5 and 8 are likely to form a salt bridge, and [D-Orn^5,Glu^8] was adapted for synthetic convenience rather than the [D-Glu^5,Orn^8] form. The L-Phe derivative is slightly more potent than the linear Ac-[Nle^4]α-MSH$_{(4-11)}$-NH$_2$ analogue, but less active in the lizard skin assay. The D-Phe^7 derivative is less active than the L-Phe form in both assays. NMR data obtained with these analogues suggested that close proximity of the His-Phe-Arg side-chains seems to be favourable for triggering the biological response.

Lactam formation between positions 5 and 10 in Ac-[Nle^4,$\overbrace{\text{Glu}^5}$,D-$\overbrace{\text{Phe}^7,\text{Lys}^{10}}$,Gly^{11}]α-MSH$_{(4-13)}$-NH$_2$ and Ac-[Nle^4,$\overbrace{\text{Asp}^5,\text{D-Phe}^7,\text{Lys}^{10}}$,Gly^{11}]α-MSH$_{(4-13)}$-NH$_2$ is possible whilst retaining the biological properties of the peptide (Al-Obeidi *et al.*, 1989b). This ring structure contains 23 atoms; the C-terminus is not required for the biological action of these derivatives as experiments with the 4-10 form have shown. The influence of the N-terminal amino acids has not been tested, but it is possible to add fatty acid to the N-terminus of the Ac-[$\overbrace{\text{Asp}^5}$,D-$\overbrace{\text{Phe}^7,\text{Lys}^{10}}$]α-MSH$_{(5-10)}$-NH$_2$ analogue which led to peptide conjugates that exhibit 10-100 times the biological activity of α-MSH in the Cloudman S91 tyrosinase assay. Hexanoyl- and decanoyl-analogues show approximately the same activity as α-MSH, and myristoyl- and palmitoyl-derivatives 1% of its activity in the lizard skin bioassay (Al-Obeidi *et al.*, 1992).

4.5. Influence of D-amino acid replacements within the peptide core

Investigations into the stereochemistry of the α-MSH backbone were made very early on after the discovery of the peptide structure in 1957 (Harris and Lerner, 1957) together with the observation that partial racemisation increased the bioactivity of the hormone in some bioassays (Lee and Buettner-Janusch, 1963). A

systematic study was carried out in the 1960s by Yajima *et al.*, where they investigated the influence of D-amino acid replacements within the α-$MSH_{(6-10)}$ pentapeptide using an *in vitro* frog skin assay. The His-D-Phe-Arg-Trp-Gly fragment showed a greater activity than the analogue containing only L-amino acids (Yajima *et al.*, 1966). Introduction of a second D-residue is only possible in position 9 (Yajima *et al.*, 1967). D-His-D-Phe-Arg-Trp-Gly exhibited only weak MSH activity. Replacement of arginine with its D-isomer made the peptide fragment inactive contradictory to earlier findings that it was as active as the all D-form. Introduction of a D-Phe residue into this molecule seemed to restore its bioactivity, but it did not show the prolonged activity observed with the D-Phe, L-Arg derivative (Yajima *et al.*, 1965, 1967). Substituting more than two amino acids by their D-isomers as in D-His-D-Phe-D-Arg-Trp-Gly and His-D-Phe-D-Arg-D-Trp-Gly gave peptides with no MSH activity, but weak inhibitory effects when administered at the same time as the L-L-L-L-pentapeptide (Yajima *et al.*, 1965). The all D-isomer showed even stronger inhibitory effects (Yajima *et al.*, 1966).

After it was established that [Nle^4,D-Phe^7]α-MSH as a more potent, prolonged acting analogue of α-MSH, which is more resistant to enzymatic degradation and provides the opportunity for radioiodination (Sawyer *et al.*, 1980), several other studies have been carried out concerning the bioactive conformation of α-MSH. Systematic studies by Sugg *et al.* (1986) investigated the effects of D-amino acid substitution on the Ac-[Nle^4]α-$MSH_{(4-11)}NH_2$ derivative using the frog skin assay and their results are more or less in agreement with those by Yajima *et al.*, although the antagonistic effects reported in the earlier studies have not been confirmed. In addition to the biological experiments, Sugg *et al.* used NMR studies to elucidate the conformation possibly recognised by the receptor. They proposed a left-handed turn in the 6-9 portion of the peptide with a *gauche* conformation of the amino acid side-chains as the most active conformation.

Recognising the key role of Phe^7, Wilkes *et al.* (1986) then made a range of changes to this residue in the Ac-[Nle^4]α-MSH$_{(4-11)}$-NH$_2$ analogue in order to investigate the influence of this particular amino acid. They replaced phenylalanine by tyrosine, p-nitrophenylalanine, alanine and glycine as well as by their corresponding D-amino acids. In all cases, except when substituting with alanine, the D-isomers were more potent than the L-forms in both the frog and the lizard skin assay, but none of them was as potent as Ac-[Nle^4]α-MSH$_{(4-11)}$-NH$_2$ or Ac-[Nle^4,D-Phe^7]α-MSH$_{(4-11)}$-NH$_2$ in those assays. A further study by this group investigated the replacement by phenylglycine (Pgl) and 1,2,3,4-tetrahydroisoquinoline (Tic) and their D-isomers, but none of them was more potent than Ac-[Nle^4]α-MSH$_{(4-11)}$-NH$_2$ or Ac-[Nle^4,D-Phe^7]α-MSH$_{(4-11)}$-NH$_2$, except Ac-[Nle^4,Pgl^7]α-MSH$_{(4-11)}$-NH$_2$ in the frog skin assay. Replacement by D-Pgl led to a major reduction in activity in both frog and lizard bioassays whereas introduction of Tic affected the results in lizard skin significantly more than those from the frog skin assay (Hruby *et al.*, 1988). Summarising these findings it is obvious that introducing these types of conformational constraints in position 7 is not favourable for the biological activity of the peptide. Binding experiments using [^{3}H]-Ac-[Nle^4,D-Phe^7]α-MSH$_{(4-11)}$-NH$_2$ (Panasci *et al.*, 1987) and [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH (Siegrist *et al.*, 1988) revealed that the D-Phe analogues also exhibit greater binding affinities than α-MSH itself. Eberle (1988) has suggested that this phenomenon might be caused by nearly "irreversible" binding to the receptor.

Introduction of the D-amino acid in position 7 increased the activity of all α-MSH analogues reported in the literature, except for a cyclic lactam analogue of Ac-α-MSH$_{(4-11)}$-NH$_2$, [D-Orn^5,Glu^8]α-MSH$_{(4-11)}$–NH$_2$, where the L-Phe^7 analogue is more potent in the frog skin assay and in the lizard skin assay (Sugg *et al.*, 1988).

Investigations into the influence of the stereochemistry of α-MSH on its other functions have not systematically been made, but there are reports by Beckwith *et al.* (1989), that while α-MSH enhances memory reactivation in rats with hypothermia-induced amnesia, Ac-[Nle^4,D-Phe^7]α-MSH$_{(4-10)}$-NH$_2$ had an amnesic effect in some of the experiments while acting like α-MSH in some others. The cyclic compounds Ac-[$\overbrace{\text{Cys}^4,\text{Cys}^{10}}$]α-MSH$_{(4-10)}$-NH$_2$ and Ac-[$\overbrace{\text{Cys}^4,\text{D-Phe}^7,\text{Cys}^{10}}$]α-MSH$_{(4-10)}$-NH$_2$ were also shown to be less active than α-MSH in these assays.

In this work, results obtained with D-Phe^7 analogues of α-MSH were generally in agreement with findings of other groups. [Nle^4,D-Phe^7]α-MSH showed a 10.2 times higher binding affinity compared to α-MSH. Results by Siegrist *et al.* previously suggested a 6.2 times stronger receptor binding (Siegrist *et al.*, 1988). Its biological activity in the tyrosinase and melanin assay was found to be 33.9 and 7.4 times that of the native hormone, respectively. This is also in the range of values found in the literature (compare Eberle, 1988). Whether the introduction of the D-Phe^7 residue is involved in stabilising a type of reverse turn as suggested by Hruby *et al.* (1988), or indeed any other structure favoured by the receptor, is not fully clear. A three-dimensional model for the receptor would facilitate further investigation of this problem.

[Nle^4,Asp^5,D-Phe^7,Lys^{10},Arg^{11}]α-MSH is a full-length derivative of Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{(4-11)}$-NH$_2$, which was first described by Al-Obeidi *et al.* (1989a) and is of interest because lactam cyclisation is possible between positions 5 and 10. It was hoped to restore full activity of the peptide by including both terminal ends. The short linear peptide was reported to exhibit approximately the same activity as α-MSH in the frog skin assay and 8 times the activity in the lizard skin assay, but no binding data is available. In our study, the full-length linear analogue showed similar affinity and activity to the originally reported

[Nle^4,D-Phe^7]α-MSH ligand with its relative potencies being 6.38, 39.1 and 10.1 in the three assays, respectively. The additional conformational restraint imparted on the peptide by the introduction of the lysine residue instead of the glycine in position 10 did obviously not significantly affect the properties of the peptide; an effect that could be observed with the substitution of glycine by alanine in α-MSH as well (see section *3.3.*).

Replacement of L-Phe by D-Phe in position 7 of a cyclic analogue, Ac-[Cys^4,D-Phe^7,Cys^{10}]α-MSH$_{(4-13)}$-NH$_2$, increased the binding affinity and biological activity of the peptide compared to α-MSH and linear and cyclic [Cys^4,Cys^{10}]α-MSH, with the potency of the peptide now being similar to [Nle^4,D-Phe^7]α-MSH. This suggests that this cyclised compound is a very good model for the bioactive conformation of α-MSH, although it is possible that the disulfide bridge is actually opened once the peptide is exposed to the cellular enzymes (see section *4.3.*).

[Nle^4,D-Phe^7,D-Trp^9]α-MSH, synthesised in analogy to Ac-[Nle^4,D-Phe^7, D-Trp^9]α-MSH$_{(4-11)}$-NH$_2$ first described by Hruby's group (Sugg *et al.*, 1986), had not previously been assayed in any mammalian pigment cell assays. It showed biological properties similar to [Nle^4,D-Phe^7]α-MSH in the binding and the tyrosinase assay; stimulation of melanin biosynthesis was not determined. Its relative potency is close to [Nle^4,D-Phe^7]α-MSH with 7.98 times the affinity and 12.2 times the tyrosinase stimulating activity of α-MSH. There is no evidence that additional introduction of the D-Trp further increases the biological activity as compared to [Nle^4,D-Phe^7]α-MSH in mammalian melanoma cell assays.

4.6. Other MSH peptides

The binding of α-MSH with a free N- or C-terminus was also studied. For α-MSH-OH, both binding affinity and biological activity were studied; both were not

significantly different from the naturally occurring ligand. This is in agreement with earlier findings that a free carboxyl group on the C-terminus did not affect the melanotropic activity of the peptide in the frog skin assay (Medzihradsky *et al.*, 1976). The desacetyl form of α-MSH naturally occurs in dogfish, but also in mammals. In this study, only the binding affinity of this peptide was tested, mainly to compare its affinity to that on the MC3 receptor. With a dissociation constant of 15.0nM, there was no significant difference in binding affinity compared to α-MSH. This peptide was reported to exhibit only marginally less activity than α-MSH in most bioassays except the frog skin assay, were it was ten times less active than the endogenous ligand. Its affinity was previously described as 1.3nM (Eberle, 1988); a value that was found for α-MSH in the same study as well. It seems that both terminal groups of the peptide are of no importance for the binding and biological action of the peptide at least in mammalian melanocytes, and also in most reptilian and amphibian melanophores (except *Rana pipiens*).

Comparatively little data has been published on the effects of γ-MSH peptides on melanophores and melanocytes. They are generally found to exhibit very low activities. γ_1-MSH shows the highest activity of all γ-MSH peptides in the Rana pipiens bioassay. Interestingly, this activity is increased upon acetylation. Only pro-γ_1-MSH showed a higher activity, but as the material was gained from pituitary extracts, this is likely to be caused by contamination with other MSH/ACTH peptides. None of the synthetic γ-MSH peptides displayed more than 0.1% of the activity of α-MSH in any of the assays, and it has therefore been claimed that this group peptides does not contribute to the pigmentary action of POMC peptides (Eberle, 1988). In our study, γ_1-MSH bound approximately 100 times less strongly than α-MSH, which might explain the low bioactivity of the peptide. Due to a lack of material the biological activity of the compound was not tested.

4.7. Summary

The systematic study of these peptides allows a good insight into the structure-activity relationships of the interaction of MSH peptides with the murine melanoma MC1 receptor. Both terminal ends are not essential for binding or biological activity, however, the C-terminus appears to be more important than the N-terminus. The most important amino acids for both receptor binding and biological activity have clearly been identified as Met^4, His^6, Phe^7, Arg^8 and Trp^9. Two of these amino acids can be replaced by their D-isomers; introduction of the D-Phe^7 residue significantly increases the activity of the peptide. Substitution of the Trp^9 by D-Trp^9 does not further influence the properties of the peptide. The cyclic analogue, [$\overline{Cys^4,Cys^{10}}$]α-MSH, exhibits a substantial amount of affinity and activity. If the cyclised form is actually present at the receptor binding site, then this together with the increased activity of the D-Phe^7 analogue would support the hypothesis, that residues 6-9 form a kind of β-turn. In spite of having charged side-chains, the glutamic acid residue in position 5 and the lysine residue in position 11 appear not to be important for binding affinity and biological activity. Residues in these positions might act as a spacer, but do not actively participate in the interaction of the hormone with its receptor.

Binding and biological activity data are generally in good agreement (Fig. 4.1.), but there are two analogues, [Ala^6]α-MSH and [Ala^{12}]α-MSH, where the affinity was significantly lower than that of the native hormone, but the biological activity as measured by the tyrosinase assay was not.

Data from the tyrosinase and the melanin assay were usually comparable, however, the biological activity of Ac-α-$MSH_{(1-12)}$-NH2 was significantly different from α-MSH in the melanin biosynthesis assay, but not in the tyrosinase assay.

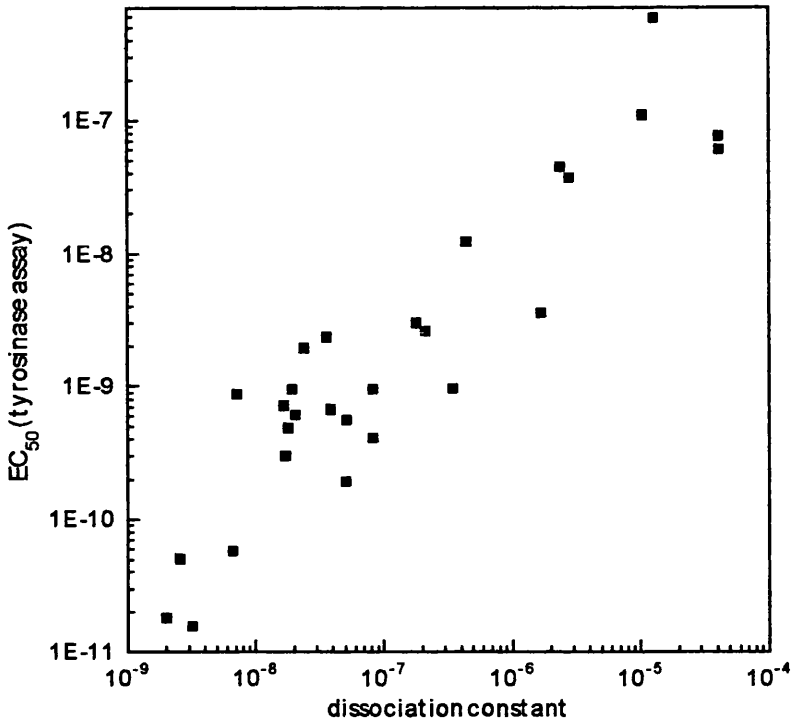


Fig. 4.1. Tyrosinase stimulation (log scale) of all α-MSH analogues tested as a function of their respective binding activities (log scale).

CHAPTER 5

Results

Binding of α-MSH, γ-MSH and of Related Peptides to the MC3 Receptor

Time-course binding experiments have been carried out with mono-iodinated [Nle^4,D-Phe^7]α-MSH to establish the equilibrium time for receptor binding at 0-4^{o}C and 37^{o}C. This was studied at 0-4^{o}C and 37^{o}C to find the optimum conditions for the binding isotherm (see section *2.4.*).

5.1. Time-course of receptor binding at 37^{o}C

Preliminary experiments in 24-well plates show that the rate of binding increases over a period of 1h. After reaching the maximum, the receptor binding decreases continuously as shown in Fig. *5.1.*.

5.2. Time-course of receptor binding at 0-4^{o}C

Preliminary experiments in 24-well plates showed a continuously increasing rate of binding over a period of 6h. Measurement after 8h indicated that although the cells had started to detach from the plates, they had not reached maximum binding. Therefore, the incubation was carried out in multiwell filtration plates which allow a 24h incubation, after which time equilibrium binding is reached (Fig. *5.2.*).

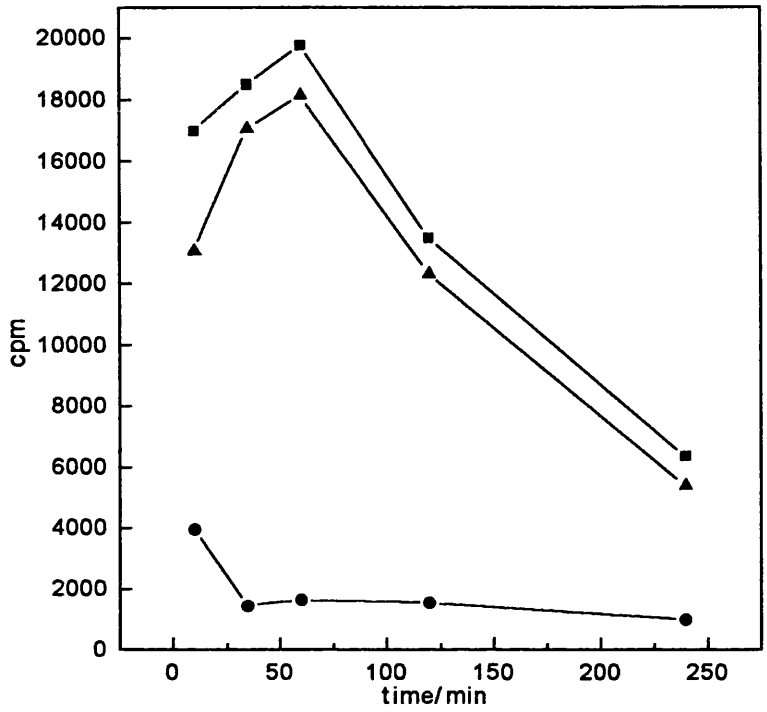


Fig. 5.1. Time-course of binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the MC3 receptor at 37^{o}C. (■) total binding, (●) non-specific binding, (▲) specific binding.

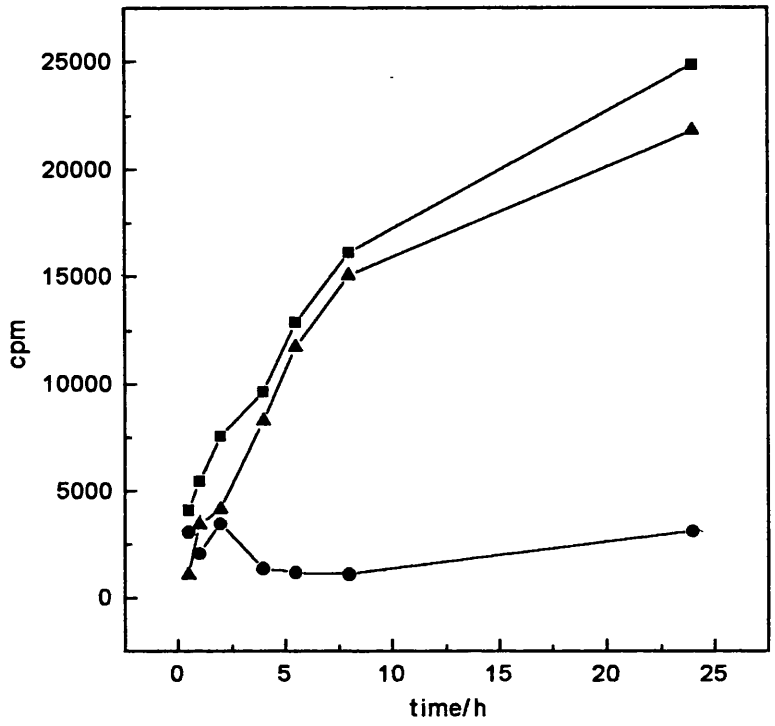


Fig. 5.2. Time-course of binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the MC3 receptor at 0-4^{o}C. (■) total binding, (●) non-specific binding, (▲) specific binding.

5.3. Binding isotherm of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

Data obtained from four replicate isotherms was analysed as described in section *2.4.2.*. An example of an isotherm is given in Fig. *5.3.*. The dissociation constant was estimated to be 1.66nM. Scatchard analysis of the binding isotherm as described in section *2.4.2.* indicated the presence of only one population of receptors (Fig. *5.4.*). The number of receptors per cell varied from 50,000 to 10,000, generally decreasing with increasing passage number. Therefore, only cells with passage numbers 10-20 were used in the binding assays.

5.4. Binding of α-MSH, desacetyl-α-MSH, [Nle^4,D-Phe^7]α-MSH and γ_1-MSH

The dissociation constants were obtained from the competition experiments as described in section *2.4.3.*. The K_d for α-MSH was determined to be 2.07±1.18μM (n=4), that for the desacetyl-analogue 0.717±0.814μM (n=3). The receptor binding of γ_1-MSH was not significantly different from that of α-MSH, with a dissociation constant of 3.24±2.15μM. [Nle^4,D-Phe^7]α-MSH bound approximately 100 times more strongly with a K_d of 21.5±17.3nM. Dissociation constants and binding relative to α-MSH are shown in Table *5.1.*. An example for the competitive binding of all three peptides is given in Fig. *5.5.*.

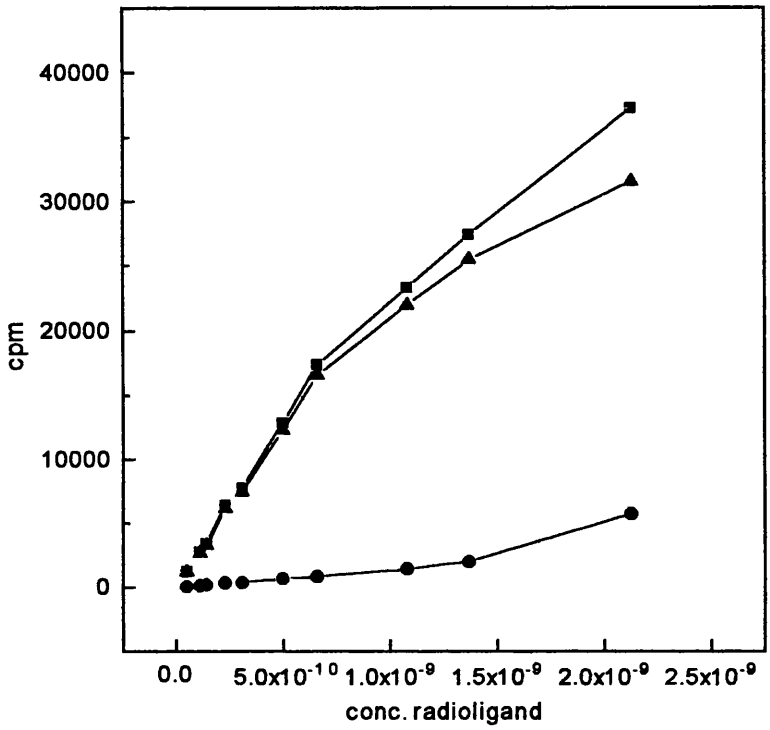


Fig. 5.3. Binding isotherm of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the MC3 receptor at 0-4°C. (■) total binding, (●) non-specific binding, (▲) specific binding.

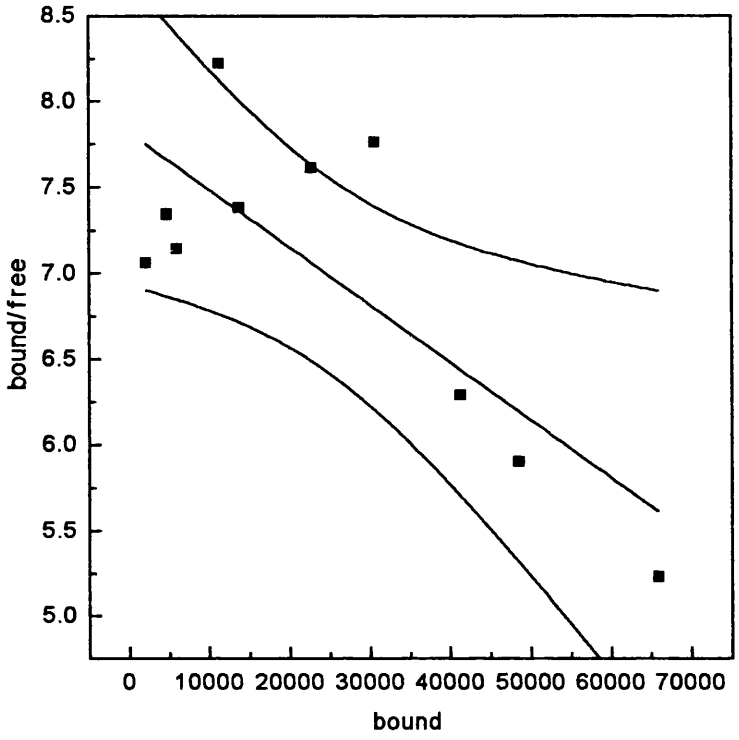


Fig. 5.4. Scatchard plot of the binding isotherm. Curved line shows 95% confidence limit.

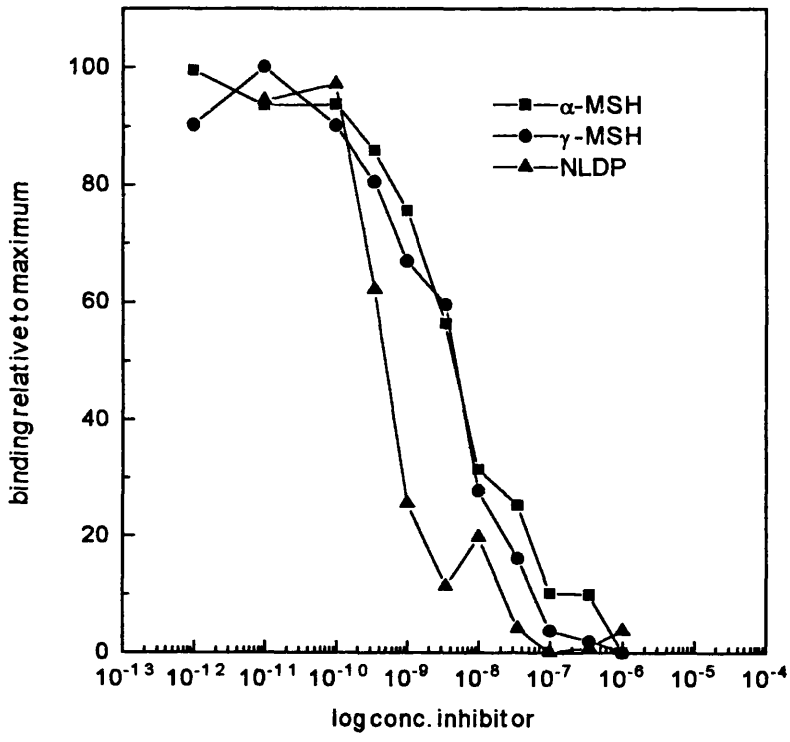


Fig. 5.5. Binding of endogenous MSH peptides and [Nle^4,D-Phe^7]α-MSH to the MC3 receptor.

Table 5.1. Dissociation constants (±SD; molar values) and relative binding affinities of MSH peptides

	Binding (K_d)	Relative Binding
[Nle^4,D-Phe^7]α-MSH	2.15×10^{-8} $\pm 1.73 \times 10^{-8}$ n=14	96.28
α-MSH	2.07×10^{-6} $\pm 1.18 \times 10^{-6}$ n=4	1
des-acetyl-α-MSH	7.17×10^{-7} $\pm 8.34 \times 10^{-7}$ n=3	2.89
γ_1-MSH	3.24×10^{-6} $\pm 2.15 \times 10^{-6}$ n=4	0.64

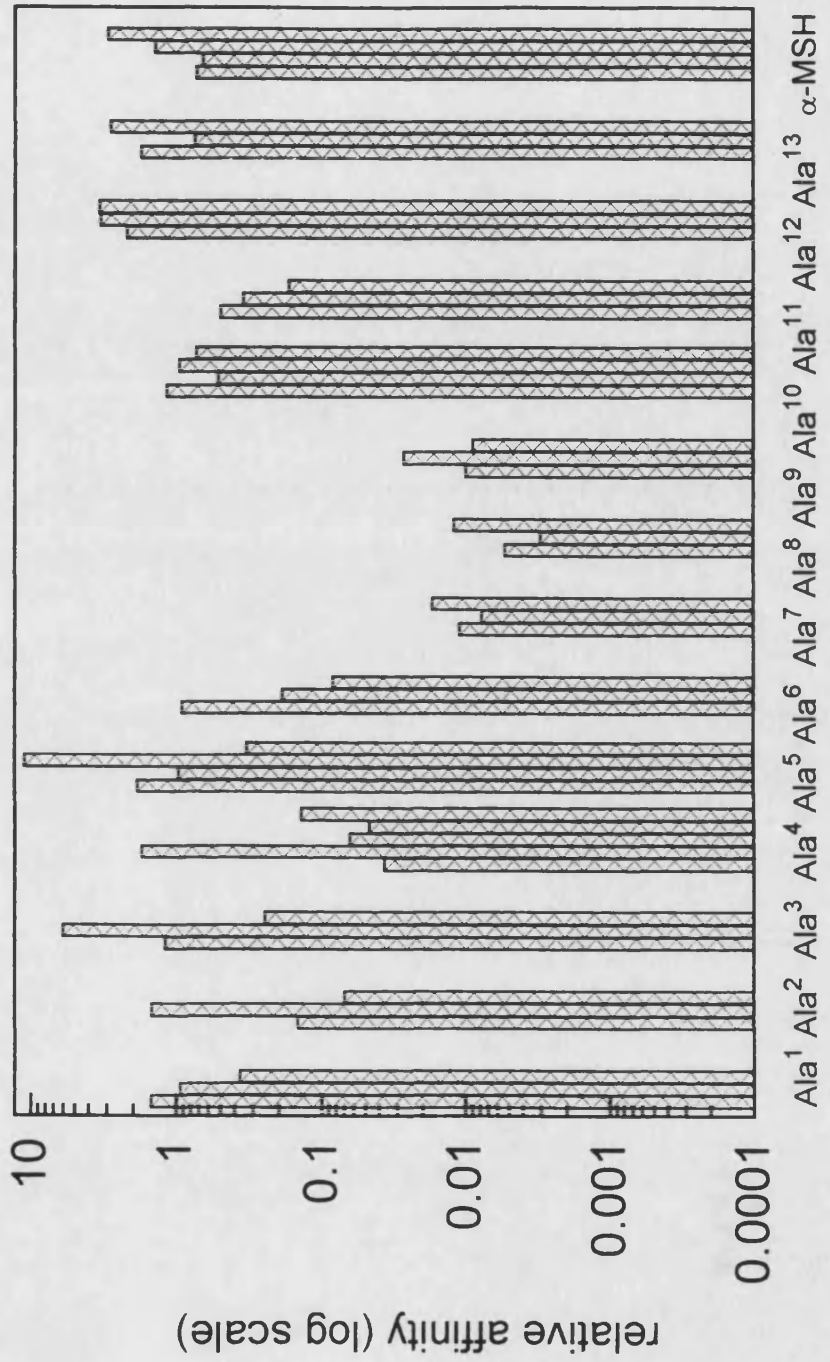
5.5. Binding of alanine substituted analogues of α-MSH

Dissociation constants were obtained as described above, and are shown in Table *5.2.*, together with values relative to α-MSH. Alanine replacements in the terminal regions of the peptide (amino acids 1-3 and 10-13) did not significantly change the binding affinity of the hormone, except for [Ala^2]α-MSH that showed only 14% of the binding of α-MSH; this difference was statistically significant at $p<0.05$. Within the peptide core -Met-Glu-His-Phe-Arg-Trp-, substitution of Glu^5 was possible without a significant loss of affinity, giving a peptide with 87% of the binding affinity of the natural compound. Replacement of Met^4 and His^6 reduced the binding to 7% and 17% of that of α-MSH, respectively. Substitution of Phe^7 and Trp^9 led to peptides with 100 times lower affinity, and the exchange of Arg^8 gave a compound with 0.0049% of the affinity of α-MSH. [Ala^7]α-MSH, [Ala^8]α-MSH and [Ala^9]α-MSH have a binding that is significantly different not only from α-MSH, but also from [Ala^4]α-MSH and [Ala^6]α-MSH (Fig. *5.6.*).

Table 5.2. Dissociation constants (±SD; molar values) of alanine analogues of α-MSH and binding relative to α-MSH

	Binding (K_d)	Relative Binding
α-MSH	$2.07x10^{-6}$ $\pm1.18x10^{-6}$ n=4	1
[Ala[1]]α-MSH	$3.08x10^{-6}$ $\pm2.28x10^{-6}$ n=3	0.67
[Ala[2]]α-MSH	$1.50x10^{-5}$ $\pm1.42x10^{-5}$ n=3	0.14
[Ala[3]]α-MSH	$3.41x10^{-6}$ $\pm4.18x10^{-6}$ n=3	0.61
[Ala[4]]α-MSH	$2.95x10^{-5}$ $\pm2.20x10^{-5}$ n=5	0.070
[Ala[5]]α-MSH	$2.39x10^{-6}$ $\pm2.63x10^{-6}$ n=4	0.87
[Ala[6]]α-MSH	$1.25x10^{-5}$ $\pm1.13x10^{-5}$ n=3	0.17
[Ala[7]]α-MSH	$1.94x\ 10^{-4}$ $\pm7.36x\ 10^{-5}$ n=3	0.011
[Ala[8]]α-MSH	$4.21x\ 10^{-4}$ $\pm2.62x\ 10^{-4}$ n=3	0.0049
[Ala[9]]α-MSH	$1.72x\ 10^{-4}$ $\pm8.42x10^{-5}$ n=3	0.012
[Ala[10]]α-MSH	$2.72x10^{-6}$ $\pm9.80x10^{-7}$ n=4	0.76
[Ala[11]]α-MSH	$7.49x10^{-6}$ $\pm4.27x10^{-6}$ n=3	0.28
[Ala[12]]α-MSH	$7.25x10^{-7}$ $\pm1.74x10^{-7}$ n=3	2.86
[Ala[13]]α-MSH	$1.57x10^{-6}$ $\pm1.08x10^{-6}$ n=3	1.32

Fig. 5.6. Relative affinities of alanine substituted α-MSH derivatives to the MC3 receptor. Individual bars represent single experiments.



CHAPTER 6

Discussion

Binding of α-MSH, γ-MSH and of Related Peptides to the MC3 Receptor

The MC3-receptor is a novel melanocortin receptor, whose biological role is as yet undiscovered, and although several MSH peptides have been tested for binding and stimulation of adenylate cyclase, no attempts have been made to study the structure-activity relationships.

A modified method for assaying the receptor binding had to be employed because the transfected cells expressing the receptor did not maintain a monolayer for the required period of time. Binding experiments carried out in 96-well filtration plates are a practical alternative to assays carried out in a monolayer or in suspension. The plates facilitate the washing procedure and allow assays to be carried out where there are problems with the detachment of cells, i.e. during long incubations or under unfavourable conditions. They also facilitate the handling of larger numbers of test points at a time. Control experiments with B16 murine melanoma cells carried out in 24-well plates and 96-well plates showed that there is no significant difference in the dissociation constant between methods. Non-specific binding to the plates was high in the absence of cells, but lower in the presence of cells. Non-specific binding to empty wells could not be reduced by excess unlabelled [Nle^4,D-Phe^7]α-MSH.

6.1. Time-course of binding

As in B16 cells, binding at 37°C reached a maximum and then constantly decreases. The time that it takes to achieve this maximum binding was significantly longer than it took with B16 cells (1h vs. 0.35h) as described by Eberle (1988) and Erskine-Grout (1993). This might be due to the higher number of receptors per cell (appr. 50,000 as compared to 10,000-20,000) or the lower affinity of the ligand, or both. Also, the ligand, once internalised, might be recycled more slowly and so appear within the cells for a longer period of time. To this time, no internalisation experiments have been carried out on these cells and they might be of limited value as the ligand does not possess a natural function in these cells. The decrease in binding after reaching the maximum is likely to be due to degradation of the receptor-ligand complex within the cell. Non-specific binding is low and does not increase with time. At 0-4°C, binding had reached equilibrium after 24h.

6.2. Binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

The dissociation constant obtained in replicate experiments was 1.66nM, thus [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH has a three times lower affinity to these receptors than to B16 α-MSH receptors. As expected, there was only one population of receptors present. The number of receptors per cell appeared to decrease with passage number, starting with >50,000 receptor per cell for passage 10 decreasing to approximately 10,000 receptors per cell with passage 21. This might be caused by a small number of cells without the gene encoding the receptor, present in the original population, that proliferate at a slightly higher rate than the transfected cells. Although a correct estimate of the receptor number is not crucial for the calculation of the affinity of the ligand, to avoid errors caused by a only small amount of specifically bound radioactivity, only cells with passage numbers between 10 and 20 were used in the binding assays.

6.3. Binding of endogenous MSH peptides and [Nle^4,D-Phe^7]α-MSH

α-MSH bound with an affinity of 2.07μM to the MC3 receptor, des-acetyl α-MSH showed a slightly lower dissociation constant of 0.717μM. γ_1-MSH showed a similar affinity, 3.24μM. [Nle^4,D-Phe^7]α-MSH bound 100 times more strongly than both natural peptides. This result is in disagreement with findings by Roselli-Rehfuss *et al.* (1993), who found a similar dissociation constant for [Nle^4,D-Phe^7]α-MSH, but significantly higher affinities for α-MSH, γ_1-MSH and γ_2-MSH. Affinities of α-MSH and γ_2-MSH reported in their study were only approximately 5 times lower than that of [Nle^4,D-Phe^7]α-MSH. These studies, however, were performed in suspension at 37°C using a significantly lower concentration of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH. There have been no investigations on the influence of the temperature on dissociation constants. At 37°C a range of problems might arise due to internalisation and/or degradation of the ligand. To avoid this, Roselli-Rehfuss *et al.*, employed a variety of protease inhibitors. The presence of bacitracin, however, has been reported to significantly reduce specific binding in B16 melanoma cells (Siegrist *et al.*, 1988) and it is possible that this might also affect the results in other cell lines. Also, a very short incubation time of 10 minutes was used in this assay, whereas our results indicate that maximum binding is only reached after more than 1h. Possibly, some variation might have been generated by use of different software for data analysis. The possibility of obtaining lower values for α-MSH and γ_1-MSH in our study due to more permanent binding of the D-Phe^7 analogues to the receptor during a 24h incubation has been eliminated by carrying out experiments with B16 cells, that showed that the K_d estimated from these experiments is similar to those obtained in a 8h incubation (see section *3.2.1.*) and to those reported in the literature (Eberle, 1988). Also, affinity values for α-MSH on melanoma cells obtained with a method similar to the one employed by Roselli-Rehfuss *et al.* were approximately ten times higher than reported before in the literature (Tatro *et al.*, 1990).

Roselli-Rehfuss *et al.* found that a whole range of MSH peptides, including [Nle^4,D-Phe^7]α-MSH, α-MSH, γ_1-MSH and γ_2-MSH, stimulated cAMP generation with EC_{50} values very similar to each other. Des-acetyl-α-MSH was the least active peptide in the cAMP assay, with an EC_{50} that was approximately 10 times lower than that of [Nle^4,D-Phe^7]α-MSH, the most active peptide. No binding data was reported for this compound, but the data reported here suggests that des-acetyl-α-MSH binds more strongly than α-MSH or γ_1-MSH. Data from the cAMP assay published by Roselli-Rehfuss *et al.* was generally in agreement with data reported for the human equivalent of this receptor, where α-MSH, β-MSH, γ-MSH, ACTH and [Nle^4,D-Phe^7]α-MSH triggered an equal biological response as measured by a cAMP assay (Gantz *et al.*, 1993a). The biological response of a peptide does not necessarily have to correspond to its receptor binding, but further studies will be needed to give an answer to this discrepancy between the results from the respective cAMP assays and the binding studies reported here.

6.4. Binding of alanine-substituted analogues of α-MSH

Because of the similar affinity of α-MSH and γ_1-MSH to this receptor, alanine analogues of α-MSH were assayed for receptor binding to investigate which amino acids are particularly important for the interaction of the peptide with this receptor. In the N-terminal part of the peptide, [Ala^1]α-MSH and [Ala^3]α-MSH did not show a significantly different dissociation constant from α-MSH. The normal residues in those positions are serine, which can be found in α-MSH and ACTH, but not in β-MSH and γ-MSH. Exchange of Tyr^2 led to reduction of the binding affinity to 14% of that of α-MSH. Tyr^2 is conserved in all MSH/ACTH sequences, and it might possibly have a role in receptor recognition. It has been proposed that the tyrosine residue is a requirement for the stimulation of the biological action on the MC4 receptor (Gantz *et al.*, 1993b), but no such investigations have been made for the MC3 receptor. Any of the C-terminal amino acids can be replaced

without significant loss of affinity, although the dissociation constant of [Ala^{11}]α-MSH is significantly different from those of [Ala^{12}]α-MSH and [Ala^{13}]α-MSH, but not from that of α-MSH. Within the core sequence, α-MSH$_{(4-9)}$, position 5 is the only one which is not conserved in all MSH/ACTH peptides, and also the only one which can be replaced by alanine without loss of binding affinity. Substitution of Met^4 and His^6 gave peptides with a similar affinity, their relative affinities being 7% and 17%, respectively. It has been observed that, as on the MC1 receptor, isosteric replacement of Met^4 by norleucine is possible without loss of receptor binding. The most important residues are clearly contained within the -Phe^7-Arg^8-Trp^9- triplet. Replacement of Phe^7 or Trp^9 lowered the affinity by 100 fold, and substitution of Arg^8 reduced the affinity by 200 fold.

Alanine analogues can be grouped according to their affinity: analogues with an affinity similar to α-MSH: [Ala^1]α-MSH, [Ala^3]α-MSH, [Ala^5]α-MSH, [Ala^{10}]α-MSH, [Ala^{11}]α-MSH, [Ala^{12}]α-MSH and [Ala^{13}]α-MSH; those with approximately 10 times lower affinity: [Ala^2]α-MSH, [Ala^4]α-MSH and [Ala^6]α-MSH; and those with 100-200 times lower receptor binding: [Ala^7]α-MSH, [Ala^8]α-MSH and [Ala^9]α-MSH. With the exception of [Ala^2]α-MSH, all replacements that led to reduced binding affinity were made within the so-called core sequence, α-MSH$_{(4-9)}$.

6.5. Summary

The significantly higher affinity of the D-Phe^7 analogues [Nle^4,D-Phe^7]α-MSH and [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH indicates that this configuration might lead to "irreversible" binding of the peptide to the receptor in a similar way to that suggested for the MC1 receptor (Eberle, 1988). Iodination of [Nle^4,D-Phe^7]α-MSH increased its affinity 10 fold, so that higher lipophilicity in this region must

lead to stronger interaction with the receptor. Again, this is very similar to observations made on the MC1 receptor.

The N-terminal acetyl group does not appear to be required by the receptor, and there is a small chance that the desacetyl form is actually the one preferred by the receptor, but, although des-acetyl-α-MSH bound with a slightly higher affinity than α-MSH itself, the data reported here is not strong enough to support this hypothesis.

In this study, there is no indication that this receptor might provide a site of action for a specific physiological role for γ-MSH as has been proposed in the literature (Roselli-Rehfuss *et al.*, 1993). Of course, not all γ-MSH peptides have been tested for receptor binding and/or biological action on this receptor and as little is known about the physiological role of those peptides or their active forms, it is quite possible that the peptides known as γ-MSHs are not actually the form required by the receptor to stimulate biological action. γ-MSH peptides bind as strongly as other melanotropic peptides to the receptor, and they trigger the biological response in the same way.

It has been suggested, that the $\alpha\text{-MSH}_{(4\text{-}10)}$-sequence is recognised by the MC3 receptor because all MSH peptides bind equally well to the receptor (Gantz *et al.*, 1993a). The results presented here are generally in agreement with that view; but in addition it has been shown, that the Glu/Gly in position 5 and the Gly/Asp in position 10 are not necessary for the receptor recognition, and that additional presence of Tyr^2 is favourable.

CHAPTER 7

Discussion

This chapter will compare the binding affinities of the various peptides studied to each other. The two series of peptides used were firstly a group of endogenous MSH peptides and the synthetic derivative [Nle^4,D-Phe^7]α-MSH and secondly the alanine-substituted analogues of α-MSH. Results for the individual receptors are described in chapters 3 and 4, and chapters 5 and 6, respectively. The amino acid sequence of the receptors will be compared, and conserved amino acids likely to interact with the ligand will be assigned.

7.1. Comparison of binding to MC1 and MC3 receptor

Binding studies could be carried out with both sets of receptors, but biological activity was only measured on the MC1 receptor, as the 293 cells transfected with the gene encoding the MC3 receptor do not possess cellular enzymes for the normal melanin biosynthetic pathway.

7.1.1. Endogenous MSH ligands and [Nle^4,D-Phe^7]α-MSH

Binding of several endogenous MSH ligands had been measured before (compare Eberle, 1988; Roselli-Rehfuss *et al.*, 1993), but there was no binding data available for γ_1-MSH on MC1 or for desacetyl-α-MSH on the MC3 receptor. As the MC3 receptor is regarded as a putative site of action of γ-MSH peptides, γ_1-MSH as the shortest compound out of this group of peptides was chosen for assay. γ_2-MSH has an extra glycine at the C-terminus, and γ_3-MSH was less active than γ_1-MSH

and γ_2-MSH in earlier studies (Roselli-Rehfuss *et al.*, 1993). On the MC1 receptor, γ_1-MSH exhibited 0.39% of the affinity of α-MSH, thus binding significantly less than both α-MSH and β-MSH. It was also reported to exhibit very little or no biological activity on the MC1 receptor in Cloudman S91 melanoma cells (Slominski *et al.*, 1992). In the work reported here, the affinity of γ_1-MSH to the MC3 receptor was of the same order of magnitude as that to the MC1, but here α-MSH showed approximately the same receptor binding as γ_1-MSH (Table *7.1.*). Compared to its action on the MC1, it retained only 1% of its affinity. Desacetyl-α-MSH was chosen as a test compound on both receptors to investigate the influence of the N-terminal acetylation, that is part of the α-MSH molecule, but not of γ_1-MSH. It appeared to influence the binding to the MC3 slightly with the desacetyl-derivative being approximately 3 times as active as α-MSH itself. However, this difference was not significant enough to fully support the hypothesis. On the MC1 receptor, desacetyl-α-MSH showed the same affinity as α-MSH. This is in agreement with values reported by Eberle (1988). [Nle^4,D-Phe^7]α-MSH binds more strongly than α-MSH to both types of receptor, but the potentiating factor is 10 for the MC1 and 100 for the MC3 receptor. Originally it was thought that this could be caused by the longer incubation times used in assays involving the MC3 receptor, but control experiments with B16 cells showed no significant difference in the dissociation constants obtained with a 8h and a 24h incubation period. However, findings by Roselli-Rehfuss *et al.* (1993) and Gantz *et al.* (1993a) suggest that the biological activity of [Nle^4,D-Phe^7]α-MSH on the MC3 is not much higher than that of α-MSH.

There is a difference in the responsiveness of each receptor to various peptides, and they also show a different sensitivity with the dissociation constants for the native ligands varying by a factor of 100. The MC1 receptor clearly prefers α-MSH as a ligand, whereas there is no pharmacological differentiation between α-MSH and γ_1-MSH on the MC3 receptor.

Table 7.1. Comparison of the relative binding affinities of endogenous MSH peptides to MC1 and MC3 receptor. Binding of α-MSH to the MC1 receptor =1.

	MC1	MC3
[Nle^4,D-Phe^7]α-MSH	10.20	0.96
α-MSH	1	0.0099
desacetyl-α-MSH	1.37	0.029
γ_1-MSH	0.0039	0.0066

7.1.2. Alanine-substituted analogues of α-MSH

As α-MSH was the preferred ligand on the MC1 receptor and showed the same affinity for the MC3 receptor as γ_1-MSH, it seemed a suitable candidate for the investigation of the structure-activity relationships on this receptor. All thirteen alanine-substituted analogues of the hormone have been tested for receptor binding. Binding to both receptors generally followed the same pattern (Fig. *7.1.*) with the N-terminal amino acids not being so important for the affinity to the receptor and the hexapeptide α-MSH$_{(4-9)}$ forming an essential "core sequence".

Within the peptide core, glutamic acid in position 5 could be replaced by alanine without affecting the binding to either receptor and thus indicating that the charged side-chain of this residue is not required for the action of the hormone. Replacement of Met^4 and His^6 reduced the affinity to both receptors significantly, but the most crucial residues were Phe^7-Arg^8-Trp^9 in both cases. The binding of the peptide to the MC1 receptor was generally more affected by amino acid substitution; this can probably be explained by its higher initial binding affinity.

In the terminal parts of the peptide, presence of the proline in position 12 was favourable to the binding on the MC1 receptor, but its absence did not affect the binding to the MC3 receptor. Proline in this position is highly conserved within melanocortin peptides: it can also be found in β-MSH and ACTH, but not in γ_1-MSH. This might partly explain the very low affinity of these MSH peptides to this particular receptor, but the influence of the residue is not strong enough to account for this on its own. Another possible way of distinguishing between the two peptides might be the aspartic acid residue in position 10 in γ_1-MSH. Although the glycine of α-MSH can be replaced by alanine without affecting the properties of the hormone, it can also be replaced by lysine (as in [Nle^4,Asp^5,D-Phe^7,Lys^{10},Arg^{11}]α-MSH; see Chapter 3), and maybe an amino acid with the opposite charge cannot be introduced into this position without loss of affinity to the MC1 receptor.

An extra requirement of the MC3 receptor is the tyrosine in position 2. This residue can however be found in all MSH/ACTH peptides, and therefore cannot exhibit any selectivity towards one of the peptides. It has been discussed that on the MC1 the tyrosine in this position might actually participate in non-specific interaction with the receptor due to its hydrophobicity (see section *4.2.*), a hypothesis that is supported by the fact that the affinity of the peptide is increased upon radioiodination, and indeed this phenomenon can be observed for the MC3 as well.

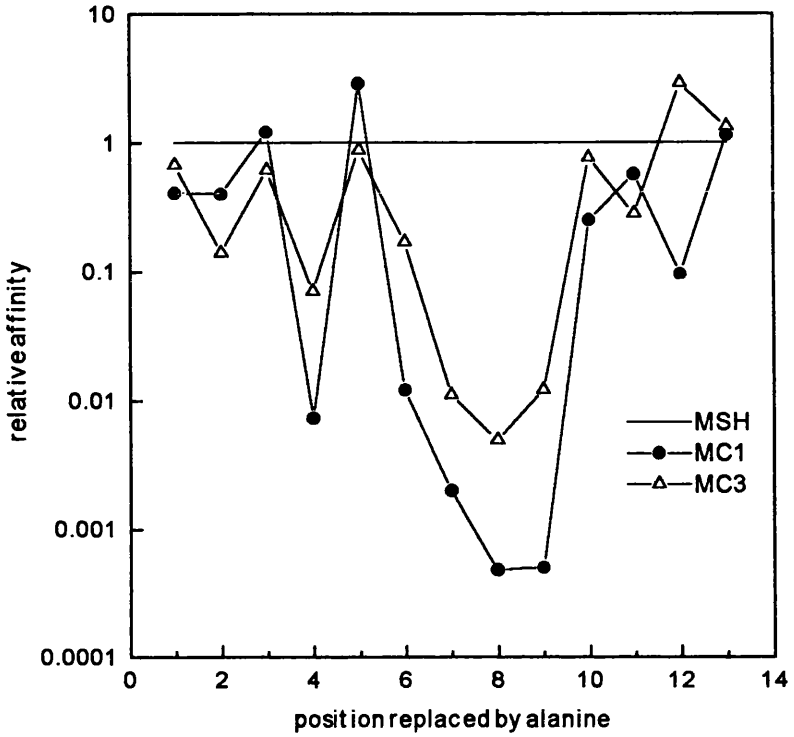


Fig. 7.1. Relative binding affinities of alanine-substituted α-MSH analogues to the MC1 and MC3 receptor.

7.2. Structural similarity of the MC1 and MC3 receptor

The seven transmembrane domains of the two receptors were assigned by hydrophobicity analysis and sequence homology (Pouton *et al.*, 1994). The amino acid sequences of the MC1 and MC3 receptor have been fitted to the universal template for G protein-coupled receptors developed by Baldwin (1993). Fig. *7.2.* shows the MC3 receptor fitted to this model. There is a ≈43% similarity between the murine melanoma MC1 and the rat hypothalamus MC3 (Roselli-Rehfuss *et al.*, 1993; see Fig. *7.3.*). They share several structural features, including the absence of a generally conserved cysteine in the first extracellular loop that is believed to form a disulfide bridge with the second extracellular loop in most G-protein coupled receptors. Also, their second extracellular loop is very short, or almost non-existent. These characteristics can also be found in the MC2 receptor (ACTH receptor; Mountjoy *et al.*, 1992). No similarities can be observed in the N-terminal region or in the extracellular parts of the receptor. There are significantly more similar features within the transmembrane regions, and the first and second intracellular loop are highly conserved. This is not surprising as these loops are generally very similar in all G protein-coupled receptors, and are thought to be important for the interaction with the G protein. The Asp^{148}-Arg^{149}-Tyr^{150} (numbering with reference to the MC3 receptor) motif on the intracellular side of helix III is thought to be crucial for G protein binding (Hargrave, 1991) and is highly conserved in all G protein-coupled receptors.

Fig. 7.2. Amino acid sequence of the MC3 receptor fitted to the Baldwin template (Baldwin, 1993)

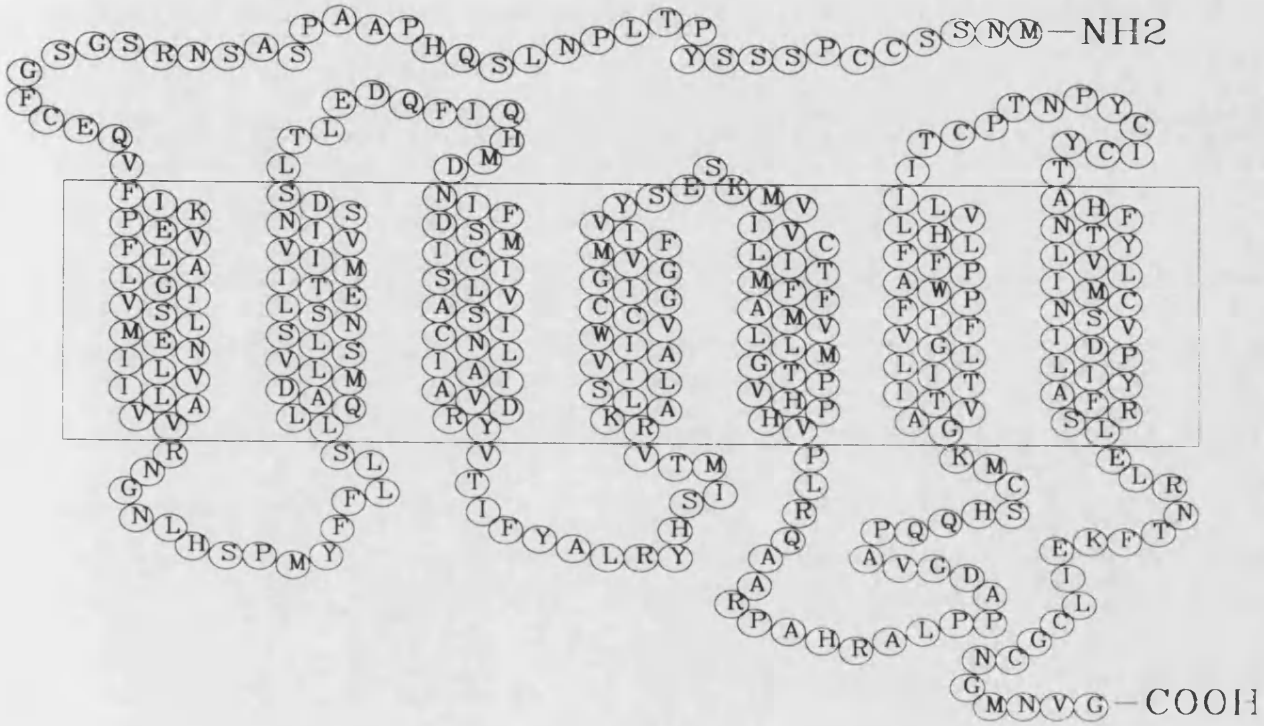
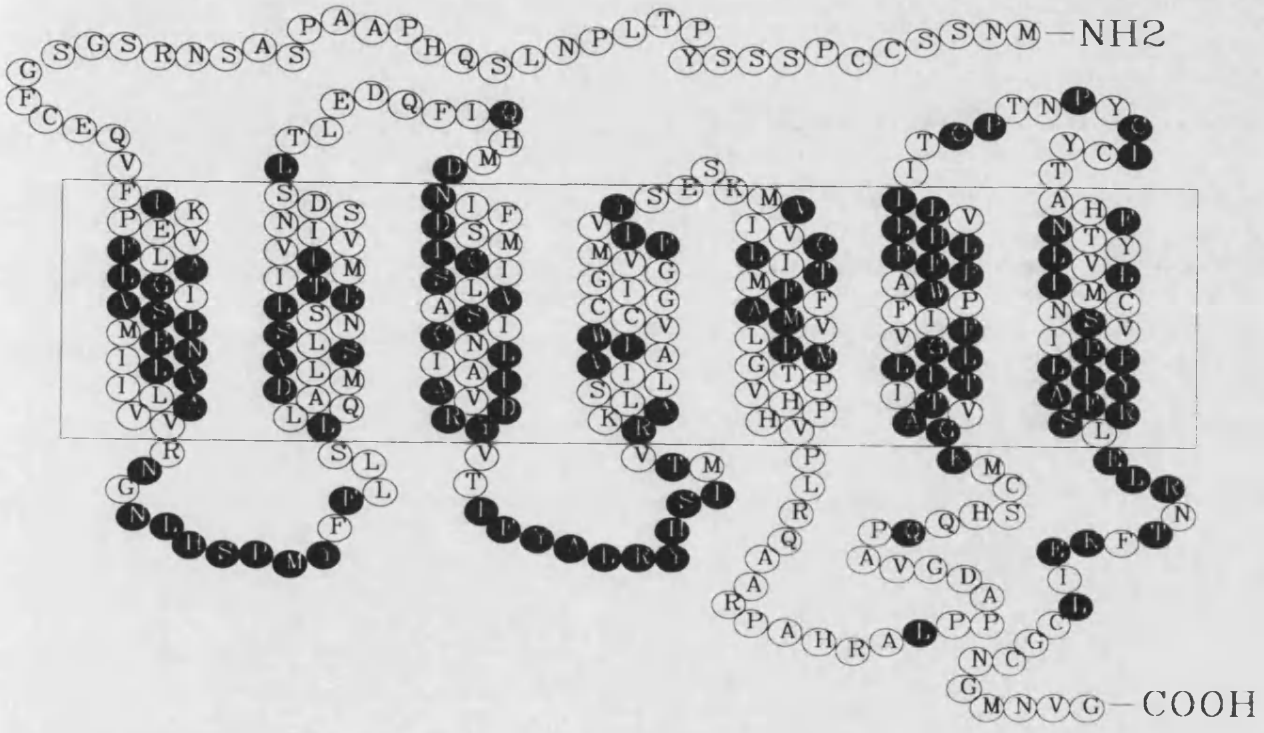


Fig. 7.3. Similarity between MC1 and MC3 receptor: common amino acids are highlighted.

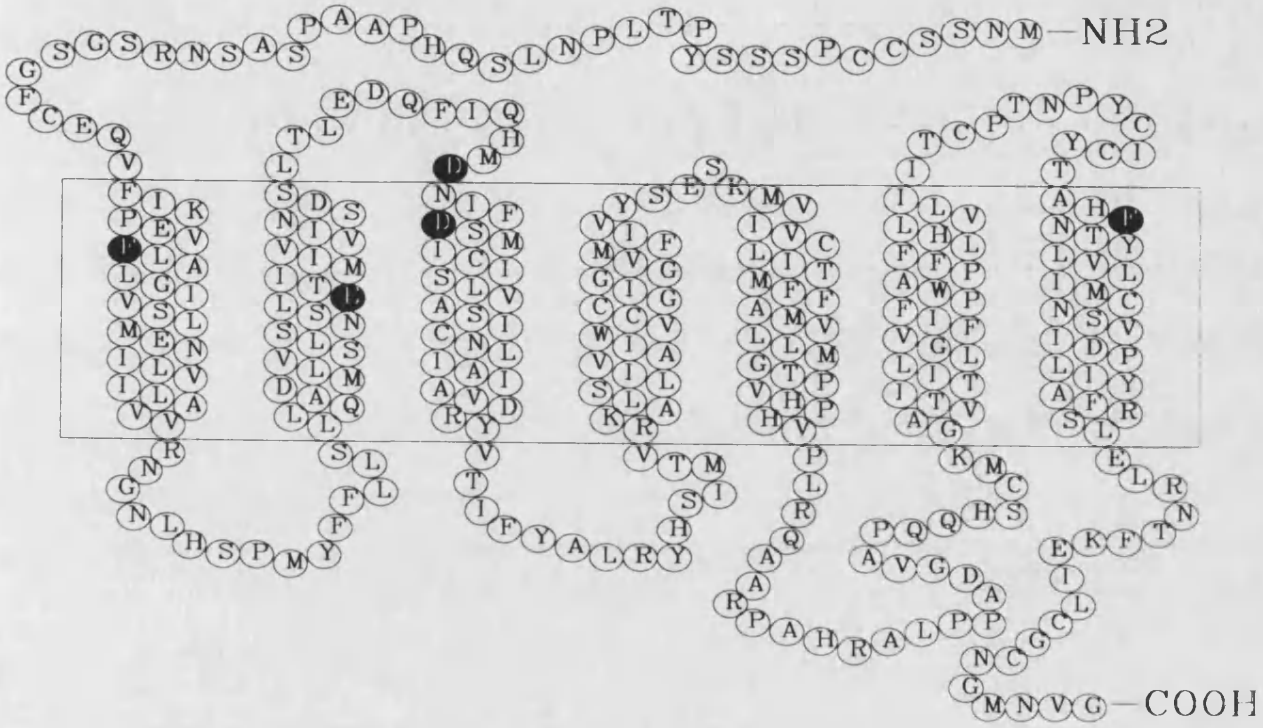


7.3. Proposed mechanism for interaction of MSH peptides with the receptors

The tetrapeptide His^6-Phe^7-Arg^8-Trp^9 is common to all melanotropic peptides and has been shown to play a major role in receptor binding and stimulation of biological activity of α-MSH. The development of a putative tertiary structure of the MSH receptor by Pouton *et al.* (1994) allows a hypothesis of the nature of the interaction of this tetrapeptide with the receptors. His^6-Phe^7-Arg^8-Trp^9- might bind to a small number of conserved residues that are located near the extracellular surfaces of the helices. These residues have been proposed to be Phe^{45}, Glu^{94}, one of Asp^{117} or Asp^{121} and Phe^{282} (numbering in reference to the MC3 receptor; see Fig. 7.4.) as there are few conserved residues that are able to interact with the ligand. This is equivalent to the amino acids in position 43, 92, 115, 119 and 277, respectively, in the MC1 receptor. The importance of the glutamate residue is known from a naturally occurring mutation on the MC1 receptor: a murine mutant, the so-called sombre mutant, shows a lysine in this position, and this receptor is constituitively active in cells cultured in the absence of the hormone (Robbins *et al.*, 1993). It was therefore suggested that the receptor might form a salt bridge with one of the aspartic acid residues within the third helix when bound to the ligand. The mutant receptor can form the bridge without the presence of MSH. The formation of the bridge might then result in a spatial rearrangement of the helices II and III, and thus trigger the response of the G protein (Pouton *et al.*, 1994).

No propositions have been made concerning the interaction of methionine, which has proven essential for the binding to both receptors, with the receptor. The mechanism of the recognition of either proline (on the MC1) or tyrosine (on the MC3) remains unsolved.

Fig. 7.4. Proposed amino acids for interaction with the His^{6}-Phe^{7}-Arg^{8}-Trp^{9} part of the MSH sequence.



7.4. Future work

The organisation of the information within the α-MSH molecule has been investigated in the work presented in this thesis. What needs to be understood next is the exact molecular interaction of the molecule with the receptor. If the proposals made concerning the binding site for the common tetrapeptide His^6-Phe^7-Arg^8-Trp^9 are correct, the influence of the other residues that are important for binding or activity can then be localised. The reason for the low binding affinity of γ-MSH peptides on the MC1 should be further investigated; the proline in position 12 of the α-MSH molecule and in the corresponding positions in β-MSH and ACTH needs to be looked at in detail. Also, the importance of the tyrosine for the binding to the MC3 is very interesting and could be further studied by replacement by amino acids other than alanine, or by peptidomimetics. To study the molecular interaction of the hormone with the ligand, point mutation experiments can be carried out with the receptor, changing one by one those residues of the receptor that are thought of importance for the binding of certain residues within the ligand molecule. Corresponding synthetic peptides can be designed that match the mutation site, and could be tested for binding and biological activity on both receptors. The question why some of the α-MSH analogues exhibit an affinity to the MC1 receptor that is significantly different from the native hormone, but nearly maintain the full biological activity remains open.

The design of conformationally constrained (i.e. cyclic) analogues with high potency remains desirable, so that conformational studies, binding and biological activity studies, and hopefully molecular modelling can be carried out simultaneously to gain information about the bioactive conformation present at the receptor.

The structure-activity studies for α-MSH on the MC1 receptor are in broad agreement with the findings in reptilian and amphibian bioassays. The central hexapeptide α-$MSH_{(4-10)}$ can be looked at as the core sequence of the peptide, however, its affinity to the murine melanoma receptor is significantly lower than that of the native hormone, and if short sequences were to be employed in drug targeting for synthetic convenience, one would have to take the loss of affinity and activity into account. Replacement of the Met^4 of Nle^4 with a drug molecule as recently suggested (Morandini *et al.*, 1994) is not advisable as this residue appears to be crucial for receptor binding. Binding to receptors within the CNS shows almost the same requirements than that to the peripheral MSH receptor in melanoma cells. Selectivity towards the latter could therefore at present not be achieved by the design of an analogue that binds specifically to one subclass of receptors.

CHAPTER 8

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APPENDIX 1

Statistical analysis of the biological data

```
1
 MTB > print c1-c4;
 SUBC> format(f2,3(f8.4)).

 1  0.1970  0.0019  0.0688
 1  0.2110  0.0016  0.0210
 1  0.1980  0.0240  0.0253
 1       *       *  0.0129
 2  2.0300  0.1390  0.1390
 2  1.5500  0.0220  0.2090
 2  2.6100  0.0110  0.1750
 2       *  0.1500       *
 3  6.3800  0.0470  0.6770
 3  4.1400  0.0670  0.2020
 3  1.0500  0.2300  0.2950
 4  2.7400  0.0073  0.6600
 4 13.7000  0.7900  0.8930
 4  8.0600  0.7000  1.9100
 4 69.9000       *       *
 4 12.5000       *       *
 5  5.3800  0.0750  0.0314
 5  1.6300  0.0510  0.1480
 5  4.5600  0.0750  0.1200
 61.23E+03 38.0000120.0000
 62.35E+03 88.0000164.0000
 6       * 50.0000532.0000
 7  2.9100  0.1020       *
 7  2.0300  0.3300  9.9000
 7  2.3300  0.3000  8.5500
 7       *       *  1.6600
 8 47.0000  1.5000 15.0000
 8 40.3000  0.2900 52.0000
 8 46.0000  1.9000 13.4000
 9266.0000  2.8000 29.4000
 9145.0000  7.7000 92.7000
 9303.0000       * 21.0000

 MTB > onew 'logbind' 'group';
1
 SUBC> fisher;
 SUBC> tukey.

 ANALYSIS OF VARIANCE ON logbind
 SOURCE     DF         SS         MS         F         p
 group       8    155.849     19.481     42.00     0.000
 ERROR      19      8.813      0.464
 TOTAL      27    164.662

                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
  LEVEL      N       MEAN      STDEV  ---------+---------+---------+-------
      1      3    -1.6000     0.0383   (--*-)
      2      3     0.7019     0.2606            (-*--)
      3      3     1.1076     0.9421             (--*-)
      4      5     2.4970     1.1687                  (-*-)
      5      3     1.2295     0.6470             (--*--)
      6      2     7.4385     0.4578                                (---*--)
      7      3     0.8740     0.1817            (--*--)
      8      3     3.7917     0.0833                       (--*-)
      9      3     5.4247     0.3933                            (--*--)
                                        ---------+---------+---------+-------
 POOLED STDEV =    0.6811                    0.0       3.0       6.0

 TUKEY'S multiple comparison procedure

         Nominal level = 0.0500
     Family error rate = 0.0500
 Individual error rate = 0.00250

 Critical value = 4.92

 Intervals for (mean of column group) - (mean of row group)

              1        2        3        4        5        6        7        8

     2   -4.237
```

```
         -0.367

     3   -4.643   -2.341
         -0.772    1.530

     4   -5.828   -3.526   -3.120
         -2.366   -0.064    0.341

1
     5   -4.765   -2.463   -2.057   -0.463
         -0.894    1.408    1.813    2.998

     6  -11.202   -8.900   -8.495   -6.924   -8.373
         -6.875   -4.573   -4.167   -2.958   -4.045

     7   -4.409   -2.107   -1.702   -0.108   -1.580    4.401
         -0.539    1.763    2.169    3.354    2.291    8.728

     8   -7.327   -5.025   -4.619   -3.026   -4.497    1.483   -4.853
         -3.456   -1.155   -0.749    0.436   -0.627    5.810   -0.982

     9   -8.960   -6.658   -6.252   -4.659   -6.130   -0.150   -6.486   -3.568
         -5.089   -2.788   -2.382   -1.197   -2.260    4.177   -2.615    0.302


 FISHER'S multiple comparison procedure

         Nominal level = 0.0500
     Family error rate = 0.505
 Individual error rate = 0.0500

 Critical value = 2.093

 Intervals for (mean of column group) - (mean of row group)

              1        2        3        4        5        6        7        8

     2   -3.466
         -1.138

     3   -3.871   -1.570
         -1.544    0.758

     4   -5.138   -2.836   -2.430
         -3.056   -0.754   -0.348

1
     5   -3.993   -1.692   -1.286    0.226
         -1.666    0.636    1.042    2.309

     6  -10.340   -8.038   -7.632   -6.134   -7.510
         -7.737   -5.435   -5.030   -3.749   -4.908

     7   -3.638   -1.336   -0.930    0.582   -0.808    5.263
         -1.310    0.992    1.397    2.664    1.519    7.866

     8   -6.556   -4.254   -3.848   -2.336   -3.726    2.345   -4.082
         -4.228   -1.926   -1.520   -0.254   -1.398    4.948   -1.754

     9   -8.189   -5.887   -5.481   -3.969   -5.359    0.713   -5.715   -2.797
         -5.861   -3.559   -3.153   -1.887   -3.031    3.315   -3.387   -0.469


 MTB > let c6 = log(c3)
 MTB > name c6 'logtyr'
 MTB > onew 'logtyr' 'group'

 ANALYSIS OF VARIANCE ON logtyr
 SOURCE     DF         SS         MS          F          p
 group       8     179.96      22.49      13.78      0.000
 ERROR      18      29.38       1.63
 TOTAL      26     209.34

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
```

```
 LEVEL      N      MEAN     STDEV  -+---------+---------+---------+-----
     1      3    -5.478     1.516   (---*----)
     2      4    -3.049     1.318           (---*---)
     3      3    -2.410     0.834             (---*----)
     4      3    -1.837     2.670              (----*---)
     5      3    -2.719     0.223            (---*----)
     6      3     4.009     0.428                                (---*----)
     7      3    -1.532     0.652                (----*---)
     8      3    -0.064     1.024                    (----*---)
     9      2     1.535     0.715                        (----*-----)
                                   -+---------+---------+---------+-----
 POOLED STDEV =    1.278          -7.0       -3.5        0.0       3.5
1
 MTB > onew 'logtyr' 'group';
 SUBC> fisher;
 SUBC> tukey.

 ANALYSIS OF VARIANCE ON logtyr
 SOURCE     DF        SS         MS         F        p
 group       8    179.96      22.49     13.78    0.000
 ERROR      18     29.38       1.63
 TOTAL      26    209.34

                                   INDIVIDUAL 95 PCT CI'S FOR MEAN
                                   BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -+---------+---------+---------+-----
     1      3    -5.478     1.516   (---*----)
     2      4    -3.049     1.318           (---*---)
     3      3    -2.410     0.834             (---*----)
     4      3    -1.837     2.670              (----*---)
     5      3    -2.719     0.223            (---*----)
     6      3     4.009     0.428                                (---*----)
     7      3    -1.532     0.652                (----*---)
     8      3    -0.064     1.024                    (----*---)
     9      2     1.535     0.715                        (----*-----)
                                   -+---------+---------+---------+-----
 POOLED STDEV =    1.278          -7.0       -3.5        0.0       3.5

 TUKEY'S multiple comparison procedure

         Nominal level = 0.0500
     Family error rate = 0.0500
 Individual error rate = 0.00254

 Critical value = 4.95

 Intervals for (mean of column group) - (mean of row group)

              1        2        3        4        5        6        7        8

     2   -5.844
          0.987

     3   -6.719   -4.054
          0.583    2.776

     4   -7.291   -4.627   -4.224
          0.011    2.203    3.078

1
     5   -6.410   -3.746   -3.342   -2.770
          0.892    3.085    3.960    4.532

     6  -13.138  -10.473  -10.070   -9.497  -10.379
         -5.836   -3.643   -2.768   -2.195   -3.077

     7   -7.597   -4.933   -4.529   -3.957   -4.838    1.890
         -0.295    1.898    2.773    3.345    2.464    9.192

     8   -9.065   -6.401   -5.998   -5.425   -6.306    0.422   -5.119
         -1.763    0.429    1.304    1.877    0.996    7.723    2.183

     9  -11.095   -8.457   -8.027   -7.455   -8.336   -1.608   -7.149   -5.681
         -2.931   -0.712    0.136    0.709   -0.172    6.555    1.015    2.483
```

```
FISHER'S multiple comparison procedure

        Nominal level = 0.0500
    Family error rate = 0.502
Individual error rate = 0.0500

Critical value = 2.101

Intervals for (mean of column group) - (mean of row group)

             1        2        3        4        5        6        7        8

    2   -4.479
        -0.378

    3   -5.259   -2.689
        -0.876    1.411

    4   -5.832   -3.262   -2.764
        -1.449    0.838    1.619

1
    5   -4.951   -2.381   -1.883   -1.310
        -0.567    1.720    2.500    3.073

    6  -11.678   -9.108   -8.611   -8.038   -8.919
        -7.295   -5.008   -4.227   -3.655   -4.536

    7   -6.138   -3.568   -3.070   -2.497   -3.379    3.349
        -1.754    0.533    1.313    1.886    1.005    7.732

    8   -7.606   -5.036   -4.538   -3.966   -4.847    1.881   -3.660
        -3.223   -0.936   -0.155    0.418   -0.464    6.264    0.723

    9   -9.464   -6.909   -6.396   -5.823   -6.705    0.023   -5.518   -4.049
        -4.563   -2.260   -1.495   -0.923   -1.804    4.924   -0.617    0.851


MTB > let c7 = log(c4)
MTB > name c7 'logmel'
MTB > onew 'logmel' 'group';
SUBC> fisher;
SUBC> tukey.

ANALYSIS OF VARIANCE ON logmel
SOURCE     DF         SS         MS         F         p
group       8    244.062     30.508     58.60     0.000
ERROR      19      9.891      0.521
TOTAL      27    253.954

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV     -----+---------+---------+---------+-
     1      4   -3.6418    0.7034     (--*-)
     2      3   -1.7606    0.2045           (--*--)
     3      3   -1.0701    0.6186              (-*--)
     4      3    0.0395    0.5475                 (--*--)
     5      3   -2.4973    0.8411          (--*--)
     6      3    5.3880    0.7853                                  (--*--)
     7      3    1.6484    0.9914                       (-*--)
     8      3    3.0848    0.7524                          (--*--)
     9      3    3.6516    0.7785                            (--*--)
                                      -----+---------+---------+---------+-
POOLED STDEV =    0.7215               -3.0       0.0       3.0       6.0

1
TUKEY'S multiple comparison procedure

        Nominal level = 0.0500
    Family error rate = 0.0500
Individual error rate = 0.00250

Critical value = 4.92

Intervals for (mean of column group) - (mean of row group)
```

	1	2	3	4	5	6	7	8
2	-3.799							
	0.036							
3	-4.489	-2.741						
	-0.654	1.360						
4	-5.599	-3.850	-3.160					
	-1.764	0.250	0.941					
5	-3.062	-1.313	-0.623	0.487				
	0.773	2.787	3.477	4.587				
6	-10.948	-9.199	-8.508	-7.399	-9.935			
	-7.112	-5.098	-4.408	-3.298	-5.835			
7	-7.208	-5.459	-4.769	-3.659	-6.196	1.689		
	-3.373	-1.359	-0.668	0.441	-2.096	5.790		
8	-8.644	-6.896	-6.205	-5.096	-7.632	0.253	-3.487	
	-4.809	-2.795	-2.105	-0.995	-3.532	4.353	0.614	
9	-9.211	-7.462	-6.772	-5.662	-8.199	-0.314	-4.053	-2.617
	-5.376	-3.362	-2.672	-1.562	-4.099	3.787	0.047	1.483

1
FISHER'S multiple comparison procedure

Nominal level = 0.0500
Family error rate = 0.505
Individual error rate = 0.0500

Critical value = 2.093

Intervals for (mean of column group) - (mean of row group)

	1	2	3	4	5	6	7	8
2	-3.0347							
	-0.7279							
3	-3.7251	-1.9235						
	-1.4183	0.5426						
4	-4.8347	-3.0331	-2.3426					
	-2.5279	-0.5670	0.1234					
5	-2.2980	-0.4963	0.1941	1.3037				
	0.0088	1.9697	2.6602	3.7698				
6	-10.1832	-8.3816	-7.6912	-6.5816	-9.1183			
	-7.8764	-5.9155	-5.2251	-4.1155	-6.6522			
7	-6.4436	-4.6420	-3.9516	-2.8420	-5.3787	2.5065		
	-4.1368	-2.1759	-1.4855	-0.3759	-2.9126	4.9726		
8	-7.8801	-6.0784	-5.3880	-4.2784	-6.8151	1.0701	-2.6695	
	-5.5733	-3.6124	-2.9219	-1.8123	-4.3491	3.5362	-0.2034	
9	-8.4468	-6.6452	-5.9548	-4.8452	-7.3819	0.5033	-3.2362	-1.7998
	-6.1400	-4.1791	-3.4887	-2.3791	-4.9158	2.9694	-0.7702	0.6663

1
MTB > stop
*** Minitab Release 7.2 *** Minitab, Inc. ***
Storage available 100000
MTB > stop
*** Minitab Release 9.1 *** Minitab Inc. ***
Worksheet size: 8000 cells

```
MTB > print c1 c2 c3 c4 c5 c6 c7

  ROW    C1        C2        C3        C4    logbind     logtyr     logmel

    1     1       1.7    0.0375   0.00585     0.5306   -3.28341   -5.14131
    2     1       7.0    0.0110   0.03010     1.9416   -4.50986   -3.50323
    3     1       6.6    0.0931   0.03020     1.8856   -2.37408   -3.49991
    4     2       6.1    0.0715   0.02530     1.8017   -2.63806   -3.67695
    5     2       4.7    0.0856   0.11200     1.5390   -2.45807   -2.18926
    6     2       4.7    0.0107   0.18300     1.5476   -4.53751   -1.69827
    7     3       2.5    0.0565   0.01500     0.8961   -2.87351   -4.19971
    8     3       1.5    0.0006   0.04080     0.4253   -7.35248   -3.19907
    9     3       1.2    0.0183   0.02680     0.1740   -4.00085   -3.61935
   10     3         *    0.0449         *          *   -3.10332          *
   11     4     408.0    1.0300   3.15000     6.0113    0.02956    1.14740
   12     4     139.0    4.6200   0.40700     4.9345    1.53039   -0.89894
   13     4     302.0    5.4200   0.70900     5.7104    1.69010   -0.34390
   14     5       1.1    0.0297         *     0.1310   -3.51661          *
   15     5       1.2    0.0373         *     0.1906   -3.28876          *
   16     5       0.5    0.1470         *    -0.7853   -1.91732          *
   17     5       0.4    0.1340         *    -0.7985   -2.00992          *
   18     5       0.3    0.4020         *    -1.0729   -0.91130          *
   19     6     308.0    0.4550   1.40000     5.7301   -0.78746    0.33647
   20     6      95.5    0.2100   0.06660     4.5591   -1.56065   -2.70905
   21     6     106.0    0.8950   0.97900     4.6634   -0.11093   -0.02122
   22     7    1220.0    7.1100   3.77000     7.1066    1.96150    1.32708
   23     7     664.0    6.8000   1.90000     6.4983    1.91692    0.64185
   24     7    1020.0   28.9000   2.31000     6.9276    3.36384    0.83725
   25     7    1300.0   20.6000         *     7.1701    3.02529          *
   26     8     306.0   10.7000         *     5.7236    2.37024          *
   27     8   77800.0    4.2900         *    11.2619    1.45629          *
   28     8    2290.0   22.2000         *     7.7363    3.10009          *
   29     8    3840.0    8.9100         *     8.2532    2.18717          *
   30     9     691.0    3.0600         *     6.5381    1.11841          *
   31     9    5790.0   10.7000         *     8.6639    2.37024          *
   32     9       5.9    0.0688         *     1.7716   -2.67655          *
   33    10       1.9    0.0163         *     0.6152   -4.11659          *
   34    10      29.9    0.0373         *     3.3979   -3.28876          *
   35    10       1.1         *         *     0.1133          *          *
   36    10       0.7    0.8190         *    -0.3467   -0.19967          *
   37    11       4.6    0.0722         *     1.5239   -2.62832          *
   38    11       2.1    0.0114         *     0.7467   -4.47414          *
   39    11       4.1    0.0389         *     1.4159   -3.24676          *
   40    11         *    0.5460         *          *   -0.60514          *
   41    12      20.7    0.3910         *     3.0301   -0.93905          *
1
   42    12      14.6    0.0978         *     2.6810   -2.32483          *
   43    12      28.8    0.0065         *     3.3604   -5.03135          *
   44    13       1.8    0.0007         *     0.5596   -7.33700          *
   45    13       1.2    0.0579         *     0.1655   -2.84904          *
   46    13       2.5    0.0102         *     0.9163   -4.58537          *
   47    13         *    0.0159         *          *   -4.14144          *
   48    13         *    0.1550         *          *   -1.86433          *
   49    14       2.0    0.0220   0.13900     0.7080   -3.81671   -1.97328
   50    14       1.5    0.0110   0.20900     0.4383   -4.50986   -1.56542
   51    14       2.6    0.1500   0.17500     0.9594   -1.89712   -1.74297
   52    14         *         *   0.26300          *          *   -1.33560
   53    14         *         *   0.25900          *          *   -1.35093

MTB > onew 'logbind' c1;
SUBC> fisher
* NOTE * Subcommand does not end in . or ; (; assumed).
SUBC> fisher;
SUBC> fisher 0.01;
SUBC> tukey.

ANALYSIS OF VARIANCE ON logbind
SOURCE     DF        SS         MS         F         p
C1         13    375.87      28.91     17.41     0.000
ERROR      33     54.81       1.66
TOTAL      46    430.68

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -----+---------+---------+---------+-
```

1	3	1.453	0.799	(---*---)
2	3	1.629	0.149	(----*---)
3	3	0.498	0.367	(---*----)
4	3	5.552	0.556	(---*---)
5	5	-0.467	0.585	(---*--)
6	3	4.984	0.648	(---*----)
7	4	6.926	0.303	(---*---)
8	4	8.244	2.289	(---*--)
9	3	5.658	3.529	(---*---)
10	4	0.945	1.682	(---*--)
11	3	1.229	0.421	(----*---)
12	3	3.024	0.340	(----*---)
13	3	0.547	0.376	(----*---)
14	3	0.702	0.261	(---*---)

POOLED STDEV = 1.289 -----+---------+---------+---------+-
0.0 3.5 7.0 10.5

1
Tukey's pairwise comparisons

Family error rate = 0.0500
Individual error rate = 0.000993

Critical value = 5.11

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5	6	7
2	-3.979 3.625						
3	-2.848 4.756	-2.671 4.933					
4	-7.902 -0.297	-7.725 -0.120	-8.856 -1.251				
5	-1.481 5.320	-1.304 5.497	-2.435 4.366	2.618 9.420			
6	-7.334 0.271	-7.157 0.447	-8.288 -0.684	-3.234 4.370	-8.852 -2.050		
7	-9.030 -1.916	-8.853 -1.740	-9.984 -2.871	-4.930 2.183	-10.517 -4.269	-5.498 1.615	
8	-10.348 -3.234	-10.171 -3.058	-11.302 -4.189	-6.248 0.865	-11.835 -5.587	-6.816 0.297	-4.611 1.975
9	-8.008 -0.403	-7.831 -0.226	-8.962 -1.357	-3.908 3.696	-9.526 -2.724	-4.476 3.129	-2.289 4.824
10	-3.049 4.064	-2.872 4.241	-4.003 3.110	1.050 8.164	-4.536 1.712	0.483 7.596	2.688 9.274
11	-3.578 4.026	-3.402 4.203	-4.533 3.072	0.521 8.126	-5.097 1.705	-0.047 7.558	2.140 9.254
12	-5.374 2.231	-5.197 2.408	-6.328 1.277	-1.274 6.330	-6.892 -0.090	-1.842 5.763	0.345 7.458
1 13	-2.897 4.708	-2.720 4.885	-3.851 3.754	1.203 8.807	-4.415 2.387	0.635 8.239	2.822 9.935
14	-3.052 4.553	-2.875 4.730	-4.006 3.599	1.048 8.652	-4.570 2.232	0.480 8.085	2.667 9.780

	8	9	10	11	12	13
9	-0.971 6.143					

10	4.006	1.156					
	10.592	8.270					
11	3.458	0.627	-3.841				
	10.572	8.231	3.273				
12	1.663	-1.168	-5.636	-5.597			
	8.777	6.436	1.478	2.007			
13	4.140	1.308	-3.159	-3.121	-1.326		
	11.253	8.913	3.954	4.484	6.279		
14	3.985	1.154	-3.314	-3.275	-1.480	-3.957	
	11.099	8.758	3.800	4.329	6.124	3.648	

Fisher's pairwise comparisons

Family error rate = 0.305
Individual error rate = 0.0100

Critical value = 2.733

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5	6	7
2	-3.053						
	2.699						
3	-1.922	-1.745					
	3.830	4.007					
4	-6.975	-6.799	-7.930				
	-1.224	-1.047	-2.178				
5	-0.653	-0.476	-1.607	3.447			
	4.492	4.669	3.538	8.591			
1							
6	-6.408	-6.231	-7.362	-2.308	-8.024		
	-0.656	-0.479	-1.610	3.444	-2.879		
7	-8.163	-7.986	-9.117	-4.064	-9.755	-4.632	
	-2.783	-2.606	-3.737	1.317	-5.030	0.749	
8	-9.481	-9.304	-10.435	-5.382	-11.074	-5.950	-3.809
	-4.101	-3.924	-5.055	-0.002	-6.348	-0.569	1.172
9	-7.081	-6.904	-8.035	-2.982	-8.697	-3.550	-1.422
	-1.329	-1.153	-2.284	2.770	-3.553	2.202	3.958
10	-2.182	-2.006	-3.137	1.917	-3.775	1.349	3.490
	3.198	3.375	2.244	7.297	0.951	6.729	8.471
11	-2.652	-2.475	-3.606	1.447	-4.268	0.880	3.007
	3.100	3.277	2.146	7.199	0.876	6.631	8.387
12	-4.447	-4.270	-5.401	-0.348	-6.063	-0.916	1.212
	1.305	1.481	0.351	5.404	-0.919	4.836	6.592
13	-1.970	-1.794	-2.925	2.129	-3.586	1.561	3.688
	3.781	3.958	2.827	7.881	1.558	7.313	9.069
14	-2.125	-1.948	-3.079	1.974	-3.741	1.406	3.534
	3.627	3.803	2.672	7.726	1.403	7.158	8.914

	8	9	10	11	12	13
9	-0.104					
	5.276					
10	4.808	2.023				

```
            9.789     7.403

     11     4.325     1.553    -2.974
            9.705     7.305     2.406

     12     2.530    -0.242    -4.769    -4.671
            7.910     5.510     0.611     1.081

1
     13     5.006     2.235    -2.292    -2.194    -0.399
           10.387     7.987     3.088     3.558     5.353

     14     4.852     2.080    -2.447    -2.349    -0.554    -3.031
           10.232     7.832     2.933     3.403     5.198     2.721


 MTB > onew 'logtyr' c1;
 SUBC> fisher;
 SUBC> fisher 0.01;
 SUBC> tukey.

 ANALYSIS OF VARIANCE ON logtyr
 SOURCE     DF        SS        MS        F        p
 C1         13    267.16     20.55     8.41    0.000
 ERROR      36     87.93      2.44
 TOTAL      49    355.09
                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
  LEVEL      N      MEAN      STDEV  ----------+---------+---------+------
      1      3    -3.389      1.072     (-----*-----)
      2      3    -3.211      1.152     (-----*-----)
      3      4    -4.333      2.071  (-----*-----)
      4      3     1.083      0.916                    (-----*-----)
      5      5    -2.329      1.074          (---*----)
      6      3    -0.820      0.725             (-----*-----)
      7      4     2.567      0.738                       (-----*----)
      8      4     2.278      0.675                     (-----*----)
      9      3     0.271      2.628                (-----*-----)
     10      3    -2.535      2.064        (------*-----)
     11      4    -2.739      1.616         (----*----)
     12      3    -2.765      2.081        (-----*-----)
     13      5    -4.155      2.077    (----*----)
     14      3    -3.408      1.353     (-----*-----)
                                      ----------+---------+---------+------
 POOLED STDEV =    1.563                    -3.0       0.0       3.0

1
 Tukey's pairwise comparisons

     Family error rate = 0.0500
 Individual error rate = 0.000975

 Critical value = 5.08

 Intervals for (column level mean) - (row level mean)

                1         2         3         4         5         6         7

      2    -4.762
            4.406

      3    -3.344    -3.166
            5.231     5.409

      4    -9.056    -8.878    -9.704
            0.111     0.289    -1.128

      5    -5.160    -4.982    -5.770    -0.688
            3.039     3.217     1.762     7.512

      6    -7.153    -6.975    -7.801    -2.681    -5.609
            2.014     2.192     0.775     6.487     2.591

      7   -10.244   -10.066   -10.869    -5.771    -8.662    -7.674
           -1.668    -1.490    -2.930     2.804    -1.130     0.901
```

8	-9.955	-9.777	-10.581	-5.483	-8.373	-7.386	-3.681
	-1.380	-1.202	-2.641	3.093	-0.841	1.190	4.258
9	-8.244	-8.066	-8.891	-3.771	-6.699	-5.674	-1.992
	0.924	1.102	-0.316	5.396	1.500	3.493	6.584
10	-5.438	-5.260	-6.085	-0.965	-3.894	-2.868	0.814
	3.730	3.908	2.490	8.202	4.306	6.299	9.390
11	-4.938	-4.760	-5.564	-0.466	-3.356	-2.369	1.336
	3.637	3.815	2.376	8.110	4.176	6.207	9.275
12	-5.208	-5.030	-5.855	-0.735	-3.664	-2.638	1.044
	3.960	4.138	2.720	8.432	4.536	6.529	9.620

1

13	-3.333	-3.156	-3.943	1.139	-1.724	-0.764	2.956
	4.866	5.044	3.589	9.339	5.377	7.436	10.488
14	-4.565	-4.387	-5.212	-0.092	-3.021	-1.996	1.687
	4.603	4.780	3.363	9.075	5.179	7.172	10.262

	8	9	10	11	12	13
9	-2.280					
	6.295					
10	0.526	-1.778				
	9.101	7.389				
11	1.047	-1.278	-4.084			
	8.987	7.297	4.491			
12	0.756	-1.548	-4.354	-4.261		
	9.331	7.620	4.814	4.314		
13	2.668	0.326	-2.479	-2.349	-2.709	
	10.200	8.526	5.720	5.183	5.490	
14	1.399	-0.905	-3.711	-3.618	-3.941	-4.847
	9.974	8.262	5.457	4.957	5.227	3.352

Fisher's pairwise comparisons

Family error rate = 0.309
Individual error rate = 0.0100

Critical value = 2.719

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5	6	7
2	-3.648						
	3.292						
3	-2.302	-2.124					
	4.189	4.367					
4	-7.942	-7.764	-8.661				
	-1.003	-0.825	-2.170				
5	-4.164	-3.986	-4.854	0.309			
	2.043	2.221	0.847	6.515			

1

6	-6.039	-5.861	-6.758	-1.567	-4.612		
	0.900	1.078	-0.267	5.373	1.594		
7	-9.202	-9.024	-9.904	-4.729	-7.746	-6.632	
	-2.710	-2.533	-3.895	1.762	-2.045	-0.141	

```
      8    -8.913    -8.735    -9.616    -4.441    -7.458    -6.344    -2.716
           -2.422    -2.244    -3.606     2.050    -1.757     0.147     3.293

      9    -7.129    -6.952    -7.849    -2.657    -5.703    -4.560    -0.949
           -0.190    -0.012    -1.358     4.282     0.504     2.379     5.542

     10    -4.324    -4.146    -5.043     0.149    -2.897    -1.754     1.856
            2.615     2.793     1.448     7.088     3.310     5.185     8.347

     11    -3.896    -3.718    -4.599     0.576    -2.441    -1.327     2.301
            2.595     2.773     1.411     7.067     3.260     5.164     8.310

     12    -4.094    -3.916    -4.813     0.379    -2.667    -1.524     2.086
            2.846     3.023     1.678     7.318     3.540     5.415     8.577

     13    -2.337    -2.159    -3.028     2.135    -0.861     0.232     3.872
            3.870     4.048     2.673     8.342     4.514     6.439     9.573

     14    -3.451    -3.273    -4.170     1.022    -2.024    -0.881     2.729
            3.488     3.666     2.321     7.961     4.182     6.058     9.220


                8         9        10        11        12        13

      9    -1.238
            5.253

     10     1.568    -0.664
            8.059     6.275

     11     2.012    -0.236    -3.042
            8.022     6.255     3.449

     12     1.798    -0.434    -3.240    -3.219
            8.289     6.505     3.700     3.272

1
     13     3.583     1.323    -1.483    -1.434    -1.713
            9.284     7.529     4.724     4.267     4.494

     14     2.441     0.209    -2.597    -2.576    -2.827    -3.851
            8.932     7.148     4.342     3.915     4.112     2.356


MTB >
MTB > onew 'logmel' c1;
SUBC> fisher;
SUBC> fisher 0.01;
SUBC> tukey.

ANALYSIS OF VARIANCE ON logmel
SOURCE     DF         SS         MS         F         p
C1          6     61.900     10.317     12.95     0.000
ERROR      16     12.745      0.797
TOTAL      22     74.645

                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL      N       MEAN      STDEV  ------+---------+---------+---------+
     1      3    -4.0482     0.9467  (-----*----)
     2      3    -2.5215     1.0303         (----*-----)
     3      3    -3.6727     0.5024    (-----*----)
     4      3    -0.0318     1.0583                   (-----*----)
     6      3    -0.7979     1.6647                 (----*----)
     7      3     0.9354     0.3530                        (-----*----)
    14      5    -1.5936     0.2705              (---*---)
                                        ------+---------+---------+---------+
POOLED STDEV =    0.8925                -4.0      -2.0       0.0       2.0

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.00405

Critical value = 4.74
```

```
Intervals for (column level mean) - (row level mean)

               1         2         3         4         6         7

     2   -3.9691
          0.9158

     3   -2.8179   -1.2912
          2.0670    3.5937

     4   -6.4588   -4.9321   -6.0833
         -1.5739   -0.0472   -1.1984

     6   -5.6927   -4.1660   -5.3172   -1.6763
         -0.8078    0.7189   -0.4323    3.2086

1
     7   -7.4260   -5.8993   -7.0506   -3.4097   -4.1758
         -2.5411   -1.0144   -2.1657    1.4752    0.7091

    14   -4.6391   -3.1124   -4.2637   -0.6228   -1.3889    0.3444
         -0.2699    1.2567    0.1055    3.7464    2.9803    4.7136


Fisher's pairwise comparisons

    Family error rate = 0.110
Individual error rate = 0.0100

Critical value = 2.921

Intervals for (column level mean) - (row level mean)

               1         2         3         4         6         7

     2   -3.6553
          0.6019

     3   -2.5040   -0.9774
          1.7532    3.2798

     4   -6.1449   -4.6183   -5.7695
         -1.8877   -0.3611   -1.5123

     6   -5.3788   -3.8522   -5.0034   -1.3625
         -1.1216    0.4050   -0.7462    2.8947

     7   -7.1121   -5.5855   -6.7367   -3.0958   -3.8619
         -2.8549   -1.3283   -2.4795    1.1614    0.3953

    14   -4.3584   -2.8317   -3.9829   -0.3421   -1.1082    0.6252
         -0.5506    0.9760   -0.1752    3.4657    2.6996    4.4329


1
MTB > onew 'logbind' c1;
SUBC> fisher.

ANALYSIS OF VARIANCE ON logbind
SOURCE     DF        SS        MS         F        p
C1         13    375.87     28.91     17.41    0.000
ERROR      33     54.81      1.66
TOTAL      46    430.68
                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -----+---------+---------+---------+-
     1      3     1.453     0.799        (---*---)
     2      3     1.629     0.149        (----*---)
     3      3     0.498     0.367     (---*----)
     4      3     5.552     0.556                    (---*---)
     5      5    -0.467     0.585  (---*--)
     6      3     4.984     0.648                  (---*----)
     7      4     6.926     0.303                        (---*---)
```

8	4	8.244	2.289	(---*--)
9	3	5.658	3.529	(---*---)
10	4	0.945	1.682	(---*--)
11	3	1.229	0.421	(----*---)
12	3	3.024	0.340	(----*---)
13	3	0.547	0.376	(----*---)
14	3	0.702	0.261	(---*---)

POOLED STDEV = 1.289

0.0 3.5 7.0 10.5

Fisher's pairwise comparisons

Family error rate = 0.736
Individual error rate = 0.0500

Critical value = 2.035

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5	6	7
2	-2.318 1.965						
3	-1.187 3.096	-1.010 3.272					
4	-6.241 -1.958	-6.064 -1.781	-7.195 -2.912				
5	0.004 3.835	0.181 4.012	-0.950 2.881	4.104 7.934			
1							
6	-5.673 -1.390	-5.496 -1.213	-6.627 -2.344	-1.574 2.709	-7.367 -3.536		
7	-7.476 -3.470	-7.299 -3.293	-8.430 -4.424	-3.377 0.630	-9.152 -5.633	-3.945 0.062	
8	-8.794 -4.788	-8.617 -4.611	-9.748 -5.742	-4.695 -0.689	-10.470 -6.951	-5.263 -1.256	-3.173 0.536
9	-6.347 -2.064	-6.170 -1.887	-7.301 -3.018	-2.247 2.036	-8.040 -4.210	-2.815 1.468	-0.735 3.271
10	-1.495 2.511	-1.319 2.688	-2.450 1.557	2.604 6.610	-3.171 0.347	2.036 6.042	4.126 7.835
11	-1.918 2.365	-1.741 2.542	-2.872 1.411	2.182 6.465	-3.611 0.220	1.614 5.897	3.694 7.700
12	-3.713 0.570	-3.536 0.747	-4.667 -0.384	0.387 4.670	-5.406 -1.576	-0.181 4.102	1.899 5.905
13	-1.236 3.047	-1.059 3.224	-2.190 2.093	2.864 7.146	-2.929 0.901	2.296 6.578	4.375 8.382
14	-1.391 2.892	-1.214 3.069	-2.345 1.938	2.709 6.992	-3.084 0.746	2.141 6.424	4.221 8.227

	8	9	10	11	12	13
9	0.583 4.589					
10	5.444 9.153	2.710 6.716				
11	5.012 9.018	2.288 6.570	-2.287 1.719			
12	3.217 7.223	0.493 4.775	-4.082 -0.076	-3.936 0.346		

```
1
     13      5.694      2.969     -1.605     -1.460      0.335
             9.700      7.252      2.401      2.823      4.618

     14      5.539      2.815     -1.760     -1.614      0.181     -2.296
             9.545      7.097      2.246      2.668      4.463      1.987


 MTB > onew 'logtyr' c1;
 SUBC> fisher.

 ANALYSIS OF VARIANCE ON logtyr
 SOURCE     DF         SS         MS         F         p
 C1         13     267.16      20.55      8.41     0.000
 ERROR      36      87.93       2.44
 TOTAL      49     355.09

                                       INDIVIDUAL 95 PCT CI'S FOR MEAN
                                       BASED ON POOLED STDEV
  LEVEL      N       MEAN      STDEV  ----------+---------+---------+------
      1      3     -3.389      1.072      (-----*-----)
      2      3     -3.211      1.152      (-----*-----)
      3      4     -4.333      2.071   (-----*----)
      4      3      1.083      0.916                        (-----*-----)
      5      5     -2.329      1.074           (---*----)
      6      3     -0.820      0.725              (-----*-----)
      7      4      2.567      0.738                          (-----*----)
      8      4      2.278      0.675                         (-----*----)
      9      3      0.271      2.628                   (-----*-----)
     10      3     -2.535      2.064         (------*-----)
     11      4     -2.739      1.616          (----*----)
     12      3     -2.765      2.081         (-----*-----)
     13      5     -4.155      2.077    (----*----)
     14      3     -3.408      1.353      (-----*-----)
                                       ----------+---------+---------+------
 POOLED STDEV =     1.563                    -3.0       0.0       3.0

 Fisher's pairwise comparisons

     Family error rate = 0.741
 Individual error rate = 0.0500

 Critical value = 2.028

 Intervals for (column level mean) - (row level mean)

                1         2         3         4         5         6         7

      2    -2.766
            2.410

      3    -1.477    -1.299
            3.364     3.542

      4    -7.060    -6.882    -7.837
           -1.885    -1.707    -2.995

      5    -3.375    -3.197    -4.130     1.097
            1.254     1.432     0.122     5.727

1
      6    -5.157    -4.979    -5.934    -0.685    -3.824
            0.018     0.196    -1.092     4.491     0.806

      7    -8.377    -8.199    -9.141    -3.904    -7.022    -5.807
           -3.535    -3.357    -4.658     0.937    -2.770    -0.966

      8    -8.088    -7.910    -8.852    -3.616    -6.733    -5.519    -1.953
           -3.247    -3.069    -4.370     1.226    -2.481    -0.677     2.530

      9    -6.248    -6.070    -7.024    -1.775    -4.914    -3.678    -0.125
           -1.072    -0.894    -2.183     3.400    -0.285     1.497     4.717

     10    -3.442    -3.264    -4.218     1.031    -2.108    -0.873     2.681
            1.734     1.912     0.623     6.206     2.521     4.303     7.523
```

```
     11    -3.071    -2.893    -3.835     1.401    -1.716    -0.502     3.064
            1.770     1.948     0.647     6.243     2.536     4.340     7.547

     12    -3.212    -3.034    -3.988     1.261    -1.878    -0.642     2.911
            1.964     2.142     0.853     6.436     2.751     4.533     7.753

     13    -1.548    -1.370    -2.303     2.924    -0.178     1.021     4.596
            3.081     3.259     1.949     7.553     3.831     5.650     8.848

     14    -2.569    -2.391    -3.345     1.903    -1.236     0.000     3.554
            2.607     2.785     1.496     7.079     3.394     5.176     8.395


               8         9        10        11        12        13

      9    -0.413
            4.428

     10     2.393     0.218
            7.234     5.394

     11     2.776     0.589    -2.217
            7.258     5.430     2.624

     12     2.623     0.448    -2.358    -2.394
            7.464     5.624     2.818     2.447

1
     13     4.308     2.111    -0.694    -0.709    -0.924
            8.560     6.741     3.935     3.543     3.705

     14     3.266     1.091    -1.715    -1.751    -1.945    -3.062
            8.107     6.266     3.461     3.090     3.231     1.567


MTB > onew 'logmel' c1;
SUBC> fisher.

ANALYSIS OF VARIANCE ON logmel
SOURCE     DF        SS        MS         F        p
C1          6    61.900    10.317    12.95    0.000
ERROR      16    12.745     0.797
TOTAL      22    74.645

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ------+---------+---------+---------+
     1      3   -4.0482    0.9467  (-----*----)
     2      3   -2.5215    1.0303         (----*-----)
     3      3   -3.6727    0.5024    (-----*----)
     4      3   -0.0318    1.0583                   (-----*----)
     6      3   -0.7979    1.6647                (----*----)
     7      3    0.9354    0.3530                        (-----*----)
    14      5   -1.5936    0.2705              (---*---)
                                    ------+---------+---------+---------+
POOLED STDEV =    0.8925               -4.0      -2.0       0.0       2.0

Fisher's pairwise comparisons

    Family error rate = 0.387
Individual error rate = 0.0500

Critical value = 2.120

Intervals for (column level mean) - (row level mean)

               1         2         3         4         6         7

     2   -3.0716
          0.0182

     3   -1.9203   -0.3937
          1.1695    2.6961

     4   -5.5612   -4.0346   -5.1858
```

```
          -2.4714   -0.9448   -2.0960

      6   -4.7951   -3.2685   -4.4197   -0.7788
          -1.7053   -0.1787   -1.3299    2.3110

1
      7   -6.5284   -5.0018   -6.1530   -2.5121   -3.2782
          -3.4387   -1.9120   -3.0632    0.5777   -0.1884

     14   -3.8363   -2.3096   -3.4609    0.1800   -0.5861    1.1472
          -1.0727    0.4539   -0.5973    2.9436    2.1775    3.9108


 MTR >
```

```
MTB > onew c5 c1;
SUBC> fisher 0.01;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C5
SOURCE     DF         SS         MS         F         p
C1          3     9.9366     3.3122     51.11     0.000
ERROR       9     0.5833     0.0648
TOTAL      12    10.5199

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N       MEAN      STDEV  ----------+---------+---------+------
     1      3    -1.5932     0.0282   (--*--)
     2      4    -1.1823     0.3739       (--*--)
     3      3    -1.3040     0.1151      (--*--)
     4      3     0.7019     0.2606                            (--*--)
                                      ----------+---------+---------+------
POOLED STDEV =    0.2546              -1.0       0.0       1.0

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0122

Critical value = 4.42

Intervals for (column level mean) - (row level mean)

               1         2         3

     2   -1.0185
          0.1968

     3   -0.9388   -0.4860
          0.3605    0.7294

     4   -2.9447   -2.4919   -2.6555
         -1.6454   -1.2765   -1.3562


1
Fisher's pairwise comparisons

    Family error rate = 0.0412
Individual error rate = 0.0100

Critical value = 3.251

Intervals for (column level mean) - (row level mean)

               1         2         3

     2   -1.0430
          0.2213

     3   -0.9649   -0.5104
          0.3866    0.7538

     4   -2.9708   -2.5163   -2.6816
         -1.6193   -1.2521   -1.3301


MTB > onew c5 c1;
SUBC> fisher.

ANALYSIS OF VARIANCE ON C5
SOURCE     DF         SS         MS         F         p
C1          3     9.9366     3.3122     51.11     0.000
ERROR       9     0.5833     0.0648
TOTAL      12    10.5199

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N       MEAN      STDEV  ----------+---------+---------+------
     1      3    -1.5932     0.0282   (--*--)
```

```
     2      4   -1.1823    0.3739        (--*--)
     3      3   -1.3040    0.1151      (--*--)
     4      3    0.7019    0.2606                          (--*--)
                                    ----------+---------+---------+------
POOLED STDEV =   0.2546                    -1.0       0.0       1.0

1
Fisher's pairwise comparisons

    Family error rate = 0.178
Individual error rate = 0.0500

Critical value = 2.262

Intervals for (column level mean) - (row level mean)

               1         2         3

     2   -0.8507
          0.0290

     3   -0.7594   -0.3181
          0.1810    0.5615

     4   -2.7652   -2.3240   -2.4761
         -1.8249   -1.4444   -1.5357


MTB > onew c6 c1;
SUBC> fisher 0.01;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C6
SOURCE     DF        SS         MS         F         p
C1          3     22.70       7.57      6.46     0.013
ERROR       9     10.54       1.17
TOTAL      12     33.23
                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV   ---+---------+---------+---------+---
     1      4    -6.345     0.260         (-----*-----)
     2      3    -7.016     1.310    (------*------)
     3      3    -5.859     1.272          (------*------)
     4      3    -3.408     1.353                      (------*------)
                                    ---+---------+---------+---------+---
POOLED STDEV =    1.082             -8.0       -6.0       -4.0       -2.0

1
Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0122

Critical value = 4.42

Intervals for (column level mean) - (row level mean)

               1         2         3

     2    -1.912
           3.253

     3    -3.069    -3.918
           2.097     1.604

     4    -5.520    -6.369    -5.213
          -0.355    -0.847     0.309


Fisher's pairwise comparisons

    Family error rate = 0.0412
Individual error rate = 0.0100
```

```
Critical value = 3.251

Intervals for (column level mean) - (row level mean)

               1         2         3

     2    -2.016
           3.357

     3    -3.172    -4.029
           2.201     1.715

     4    -5.624    -6.480    -5.324
          -0.251    -0.736     0.420


1
MTB > onew c6 c1;
SUBC> fisher.

ANALYSIS OF VARIANCE ON C6
SOURCE     DF         SS         MS         F         p
C1          3      22.70       7.57      6.46     0.013
ERROR       9      10.54       1.17
TOTAL      12      33.23
                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N       MEAN      STDEV  ---+---------+---------+---------+---
     1      4     -6.345      0.260       (-----*-----)
     2      3     -7.016      1.310    (------*------)
     3      3     -5.859      1.272          (------*------)
     4      3     -3.408      1.353                    (------*------)
                                      ---+---------+---------+---------+---
POOLED STDEV =     1.082              -8.0      -6.0      -4.0      -2.0

Fisher's pairwise comparisons

    Family error rate = 0.178
Individual error rate = 0.0500

Critical value = 2.262

Intervals for (column level mean) - (row level mean)

               1         2         3

     2    -1.198
           2.540

     3    -2.355    -3.155
           1.383     0.842

     4    -4.807    -5.606    -4.450
          -1.068    -1.610    -0.453


1
MTB > onew c7 c1;
SUBC> fisher 0.01;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C7
SOURCE     DF         SS         MS         F         p
C1          2      29.37      14.68      9.13     0.005
ERROR      11      17.70       1.61
TOTAL      13      47.07
                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N       MEAN      STDEV  ---------+---------+---------+-------
     1      6     -4.615      1.617     (------*------)
     2      3     -4.619      1.471  (---------*---------)
     4      5     -1.594      0.271                      (-------*-------)
                                      ---------+---------+---------+-------
POOLED STDEV =     1.269              -4.8      -3.2      -1.6
```

```
Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0206

Critical value = 3.82

Intervals for (column level mean) - (row level mean)

               1         2

     2    -2.419
           2.427

     4    -5.096    -5.528
          -0.947    -0.523


1
Fisher's pairwise comparisons

    Family error rate = 0.0249
Individual error rate = 0.0100

Critical value = 3.109

Intervals for (column level mean) - (row level mean)

               1         2

     2    -2.785
           2.792

     4    -5.410    -5.905
          -0.633    -0.145


MTB > onew c7 c1;
SUBC> fisher.

ANALYSIS OF VARIANCE ON C7
SOURCE     DF         SS         MS         F         p
C1          2      29.37      14.68      9.13     0.005
ERROR      11      17.70       1.61
TOTAL      13      47.07

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN      STDEV  ---------+---------+---------+-------
     1      6    -4.615      1.617     (------*------)
     2      3    -4.619      1.471  (---------*---------)
     4      5    -1.594      0.271                        (-------*-------)
                                      ---------+---------+---------+-------
POOLED STDEV =     1.269             -4.8      -3.2      -1.6

Fisher's pairwise comparisons

    Family error rate = 0.115
Individual error rate = 0.0500

Critical value = 2.201

Intervals for (column level mean) - (row level mean)

               1         2

     2    -1.970
           1.978

     4    -4.712    -5.064
          -1.331    -0.986


1
MTB > save'dphe.mtw'
```

```
MTB > onew c5 c1;
SUBC> fisher 0.01;
SUBC> tukey.

ANALYSIS OF VARIANCE ON C5
SOURCE     DF        SS        MS         F        p
C1          3    25.640     8.547     70.95    0.000
ERROR       8     0.964     0.120
TOTAL      11    26.604
                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ------+---------+---------+---------+
     1      3    3.4474    0.5635                             (--*--)
     2      3    2.1142    0.1116                   (--*--)
     3      3   -0.4359    0.2898  (--*--)
     4      3    0.7019    0.2606          (--*--)
                                      ------+---------+---------+---------+
POOLED STDEV =   0.3471                 0.0       1.5       3.0       4.5

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0126

Critical value = 4.53

Intervals for (column level mean) - (row level mean)

               1         2         3

     2    0.4254
          2.2409

     3    2.9756    1.6424
          4.7910    3.4578

     4    1.8378    0.5046   -2.0455
          3.6532    2.3200   -0.2301


1
 Fisher's pairwise comparisons

    Family error rate = 0.0403
Individual error rate = 0.0100

Critical value = 3.356

Intervals for (column level mean) - (row level mean)

               1         2         3

     2    0.3821
          2.2842

     3    2.9323    1.5991
          4.8343    3.5011

     4    1.7945    0.4613   -2.0888
          3.6965    2.3633   -0.1868


MTB > onew c5 c1;
SUBC> fisher.

ANALYSIS OF VARIANCE ON C5
SOURCE     DF        SS        MS         F        p
C1          3    25.640     8.547     70.95    0.000
ERROR       8     0.964     0.120
TOTAL      11    26.604
                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ------+---------+---------+---------+
     1      3    3.4474    0.5635                             (--*--)
```

```
     2      3    2.1142    0.1116                    (--*--)
     3      3   -0.4359    0.2898  (--*--)
     4      3    0.7019    0.2606          (--*--)
                                    ------+---------+---------+---------+
POOLED STDEV =   0.3471              0.0       1.5       3.0       4.5

1
 Fisher's pairwise comparisons

     Family error rate = 0.176
 Individual error rate = 0.0500

 Critical value = 2.306

 Intervals for (column level mean) - (row level mean)

                1         2         3

     2     0.6797
           1.9866

     3     3.2298    1.8966
           4.5368    3.2036

     4     2.0920    0.7588   -1.7913
           3.3990    2.0658   -0.4843


 MTB >
 MTB > onew c6 c1;
 SUBC> fisher0.01;
 SUBC> tukey.

 ANALYSIS OF VARIANCE ON C6
 SOURCE     DF        SS        MS        F        p
 C1          3    15.966     5.322     6.98    0.013
 ERROR       8     6.098     0.762
 TOTAL      11    22.064
                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
  LEVEL      N      MEAN     STDEV  ----+---------+---------+---------+--
      1      3   -2.4390    0.5404                      (-------*------)
      2      3   -2.4990    0.6428                      (------*-------)
      3      3   -5.2940    0.7153   (-------*------)
      4      3   -3.4079    1.3535                (------*-------)
                                    ----+---------+---------+---------+--
 POOLED STDEV =   0.8730             -6.0       -4.5       -3.0       -1.5

1
 Tukey's pairwise comparisons

     Family error rate = 0.0500
 Individual error rate = 0.0126

 Critical value = 4.53

 Intervals for (column level mean) - (row level mean)

                1         2         3

     2    -2.2233
           2.3434

     3     0.5717    0.5116
           5.1384    5.0783

     4    -1.3144   -1.3745   -4.1694
           3.2523    3.1922    0.3973


 Fisher's pairwise comparisons

     Family error rate = 0.0403
 Individual error rate = 0.0100
```

```
 Critical value = 3.356

 Intervals for (column level mean) - (row level mean)

                1         2         3

     2    -2.3322
           2.4523

     3     0.4628    0.4027
           5.2473    5.1872

     4    -1.4233   -1.4834   -4.2783
           3.3612    3.3011    0.5062


1
 MTB >
 MTB > onew c6 c1;
 SUBC> fisher.

 ANALYSIS OF VARIANCE ON C6
 SOURCE     DF         SS         MS         F         p
 C1          3     15.966      5.322      6.98     0.013
 ERROR       8      6.098      0.762
 TOTAL      11     22.064

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
  LEVEL      N      MEAN      STDEV  ----+---------+---------+---------+--
      1      3   -2.4390     0.5404                     (-------*------)
      2      3   -2.4990     0.6428                     (------*-------)
      3      3   -5.2940     0.7153   (-------*------)
      4      3   -3.4079     1.3535               (------*-------)
                                      ----+---------+---------+---------+--
 POOLED STDEV =    0.8730              -6.0      -4.5      -3.0      -1.5

 Fisher's pairwise comparisons

     Family error rate = 0.176
 Individual error rate = 0.0500

 Critical value = 2.306

 Intervals for (column level mean) - (row level mean)

                1         2         3

     2    -1.5837
           1.7039

     3     1.2112    1.1512
           4.4988    4.4387

     4    -0.6748   -0.7349   -3.5299
           2.6127    2.5527   -0.2423


1
 MTB > onew c7 c1;
 SUBC> fisher 0.01;
 SUBC> tukey.

 ANALYSIS OF VARIANCE ON C7
 SOURCE     DF         SS         MS         F         p
 C1          2      1.268      0.634      0.91     0.437
 ERROR       9      6.281      0.698
 TOTAL      11      7.549

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
  LEVEL      N      MEAN      STDEV  -+---------+---------+---------+-----
      1      3   -0.7837     0.8747            (-----------*-----------)
      2      4   -1.1711     1.2191         (----------*----------)
      4      5   -1.5936     0.2705   (---------*---------)
                                      -+---------+---------+---------+-----
```

```
POOLED STDEV =   0.8354          -2.40     -1.60     -0.80      0.00

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0209

Critical value = 3.95

Intervals for (column level mean) - (row level mean)

               1         2

     2   -1.3948
          2.1695

     4   -0.8942   -1.1428
          2.5140    1.9878


1
Fisher's pairwise comparisons

    Family error rate = 0.0245
Individual error rate = 0.0100

Critical value = 3.251

Intervals for (column level mean) - (row level mean)

               1         2

     2   -1.6870
          2.4617

     4   -1.1735   -1.3994
          2.7933    2.2444


MTB >
MTB > onew c7 c1;
SUBC> fisher.

ANALYSIS OF VARIANCE ON C7
SOURCE     DF        SS        MS        F        p
C1          2     1.268     0.634     0.91    0.437
ERROR       9     6.281     0.698
TOTAL      11     7.549

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -+---------+---------+---------+-----
     1      3   -0.7837    0.8747           (------------*-------------)
     2      4   -1.1711    1.2191        (----------*-----------)
     4      5   -1.5936    0.2705    (---------*----------)
                                    -+---------+---------+---------+-----
POOLED STDEV =   0.8354          -2.40     -1.60     -0.80      0.00

Fisher's pairwise comparisons

    Family error rate = 0.113
Individual error rate = 0.0500

Critical value = 2.262

Intervals for (column level mean) - (row level mean)

               1         2

     2   -1.0559
          1.8307

     4   -0.5701   -0.8451
          2.1900    1.6902
```

```
MTB > let c3=log(c1)
MTB > let c4=log(c2)
MTB > print c1 c2 c3 c4
```

ROW	C1	C2	C3	C4
1	1	137.0	0.00000	4.9200
2	1	219.0	0.00000	5.3891
3	1	567.0	0.00000	6.3404
4	2	1400.0	0.69315	7.2442
5	2	138.0	0.69315	4.9273
6	2	2970.0	0.69315	7.9963
7	3	173.0	1.09861	5.1533
8	3	34.2	1.09861	3.5322
9	3	817.0	1.09861	6.7056
10	4	118.0	1.38629	4.7707
11	4	5590.0	1.38629	8.6287
12	4	3240.0	1.38629	8.0833
13	4	4400.0	1.38629	8.3894
14	4	1450.0	1.38629	7.2793
15	5	110.0	1.60944	4.7005
16	5	213.0	1.60944	5.3613
17	5	18.5	1.60944	2.9178
18	5	145.0	1.60944	4.9767
19	6	225.0	1.79176	5.4161
20	6	107.0	1.79176	4.6728
21	6	246.0	1.79176	5.5053
22	7	19000.0	1.94591	9.8522
23	7	27000.0	1.94591	10.2036
24	7	12300.0	1.94591	9.4174
25	8	39000.0	2.07944	10.5713
26	8	69800.0	2.07944	11.1534
27	8	17600.0	2.07944	9.7757
28	9	20800.0	2.19722	9.9427
29	9	7620.0	2.19722	8.9385
30	9	23000.0	2.19722	10.0432
31	10	179.0	2.30259	5.1874
32	10	402.0	2.30259	5.9965
33	10	218.0	2.30259	5.3845
34	10	290.0	2.30259	5.6699
35	11	417.0	2.39790	6.0331
36	11	600.0	2.39790	6.3969
37	11	1230.0	2.39790	7.1148
38	12	93.7	2.48491	4.5401
39	12	62.2	2.48491	4.1304
40	12	61.5	2.48491	4.1190
41	13	118.0	2.56495	4.7707

1

42	13	279.0	2.56495	5.6312
43	13	72.9	2.56495	4.2891
44	14	288.0	2.63906	5.6630
45	14	321.0	2.63906	5.7714
46	14	148.0	2.63906	4.9972
47	14	70.5	2.63906	4.2556

```
MTB > onew c4 c1;
SUBC> fisher 0.01;
SUBC> tukey.
```

ANALYSIS OF VARIANCE ON C4

SOURCE	DF	SS	MS	F	p
C1	13	177.991	13.692	14.81	0.000
ERROR	33	30.512	0.925		
TOTAL	46	208.503			

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
1	3	5.550	0.724	(---*----)
2	3	6.723	1.600	(----*---)
3	3	5.130	1.587	(----*---)
4	5	7.430	1.572	(---*--)
5	4	4.489	1.082	(---*---)
6	3	5.198	0.457	(----*---)
7	3	9.824	0.394	(---*----)

POOLED STDEV = 0.962

8	3	10.500	0.692	(----*----)
9	3	9.641	0.611	(----*---)
10	4	5.560	0.352	(---*---)
11	3	6.515	0.550	(---*----)
12	3	4.263	0.240	(---*----)
13	3	4.897	0.680	(----*---)
14	4	5.172	0.700	(---*---)

POOLED STDEV = 0.962

5.0 7.5 10.0

Tukey's pairwise comparisons

Family error rate = 0.0500
Individual error rate = 0.000993

Critical value = 5.11

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5	6	7
2	-4.0097 1.6641						
3	-2.4174 3.2563	-1.2446 4.4291					
4	-4.4178 0.6569	-3.2451 1.8297	-4.8373 0.2375				
5	-1.5929 3.7144	-0.4201 4.8872	-2.0123 3.2950	0.6105 5.2719			
6	-2.4851 3.1886	-1.3123 4.3614	-2.9046 2.7692	-0.3052 4.7696	-3.3627 1.9446		
7	-7.1114 -1.4377	-5.9386 -0.2649	-7.5309 -1.8571	-4.9315 0.1433	-7.9890 -2.6817	-7.4632 -1.7894	
8	-7.7872 -2.1135	-6.6144 -0.9407	-8.2066 -2.5329	-5.6072 -0.5325	-8.6647 -3.3574	-8.1389 -2.4652	-3.5126 2.1611
9	-6.9286 -1.2548	-5.7558 -0.0820	-7.3480 -1.6743	-4.7486 0.3262	-7.8061 -2.4988	-7.2803 -1.6065	-2.6540 3.0197
10	-2.6634 2.6439	-1.4906 3.8167	-3.0828 2.2245	-0.4600 4.2014	-3.5273 1.3863	-3.0151 2.2922	1.6112 6.9185
11	-3.8020 1.8717	-2.6292 3.0445	-4.2214 1.4523	-1.6220 3.4527	-4.6795 0.6278	-4.1537 1.5200	0.4726 6.1463
12	-1.5502 4.1235	-0.3774 5.2963	-1.9696 3.7041	0.6298 5.7045	-2.4277 2.8795	-1.9019 3.7718	2.7244 8.3981
13	-2.1841 3.4897	-1.0113 4.6625	-2.6035 3.0703	-0.0041 5.0707	-3.0616 2.2457	-2.5358 3.1380	2.0905 7.7642
14	-2.2756 3.0316	-1.1028 4.2044	-2.6951 2.6122	-0.0722 4.5892	-3.1395 1.7741	-2.6274 2.6799	1.9989 7.3062

	8	9	10	11	12	13
9	-1.9782 3.6955					
10	2.2869 7.5942	1.4283 6.7356				
11	1.1483 6.8221	0.2897 5.9634	-3.6090 1.6983			
12	3.4001 9.0738	2.5415 8.2152	-1.3572 3.9500	-0.5851 5.0886		

1

13	2.7663	1.9076	-1.9911	-1.2189	-3.4707	
	8.4400	7.5814	3.3162	4.4548	2.2030	
14	2.6747	1.8161	-2.0690	-1.3105	-3.5623	-2.9285
	7.9820	7.1233	2.8445	3.9968	1.7450	2.3788

Fisher's pairwise comparisons

Family error rate = 0.305
Individual error rate = 0.0100

Critical value = 2.733

Intervals for (column level mean) - (row level mean)

	1	2	3	4	5	6	7
2	-3.3185						
	0.9729						
3	-1.7263	-0.5535					
	2.5651	3.7379					
4	-3.7997	-2.6269	-4.2191				
	0.0387	1.2115	-0.3807				
5	-0.9464	0.2264	-1.3658	1.1783			
	3.0679	4.2407	2.6484	4.7041			
6	-1.7940	-0.6212	-2.2134	0.3130	-2.7161		
	2.4974	3.6702	2.0780	4.1514	1.2981		
7	-6.4203	-5.2475	-6.8397	-4.3133	-7.3424	-6.7720	
	-2.1289	-0.9561	-2.5483	-0.4749	-3.3282	-2.4806	
8	-7.0960	-5.9232	-7.5154	-4.9890	-8.0182	-7.4477	-2.8215
	-2.8046	-1.6318	-3.2240	-1.1507	-4.0039	-3.1563	1.4700
9	-6.2374	-5.0646	-6.6568	-4.1304	-7.1596	-6.5891	-1.9628
	-1.9460	-0.7732	-2.3654	-0.2920	-3.1453	-2.2977	2.3286
10	-2.0169	-0.8441	-2.4363	0.1078	-2.9287	-2.3686	2.2577
	1.9974	3.1702	1.5780	3.6336	0.7878	1.6457	6.2720
11	-3.1108	-1.9380	-3.5303	-1.0038	-4.0330	-3.4626	1.1637
	1.1806	2.3534	0.7612	2.8345	-0.0187	0.8289	5.4552
12	-0.8591	0.3137	-1.2785	1.2479	-1.7812	-1.2108	3.4155
	3.4324	4.6051	3.0129	5.0863	2.2330	3.0806	7.7069

1

13	-1.4929	-0.3201	-1.9123	0.6141	-2.4151	-1.8446	2.7817
	2.7985	3.9713	2.3791	4.4525	1.5992	2.4468	7.0731
14	-1.6291	-0.4563	-2.0486	0.4956	-2.5410	-1.9809	2.6454
	2.3851	3.5579	1.9657	4.0214	1.1755	2.0334	6.6597

	8	9	10	11	12	13
9	-1.2871					
	3.0043					
10	2.9334	2.0748				
	6.9477	6.0891				
11	1.8395	0.9809	-2.9625			
	6.1309	5.2723	1.0518			
12	4.0912	3.2326	-0.7107	0.1061		
	8.3827	7.5240	3.3035	4.3975		

```
    13     3.4574     2.5988    -1.3446    -0.5278    -2.7795
           7.7488     6.8902     2.6697     3.7636     1.5119

    14     3.3212     2.4626    -1.4705    -0.6640    -2.9158    -2.2819
           7.3354     6.4768     2.2460     3.3503     1.0985     1.7323


MTB >
MTB > onew c4 c1;
SUBC> fisher.

ANALYSIS OF VARIANCE ON C4
SOURCE     DF        SS         MS         F        p
C1         13   177.991     13.692     14.81    0.000
ERROR      33    30.512      0.925
TOTAL      46   208.503
1
                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN      STDEV  --------+---------+---------+--------
     1      3     5.550      0.724        (---*----)
     2      3     6.723      1.600            (----*---)
     3      3     5.130      1.587      (----*---)
     4      5     7.430      1.572                (---*--)
     5      4     4.489      1.082    (---*---)
     6      3     5.198      0.457      (----*---)
     7      3     9.824      0.394                          (---*----)
     8      3    10.500      0.692                            (----*----)
     9      3     9.641      0.611                         (----*---)
    10      4     5.560      0.352        (---*---)
    11      3     6.515      0.550            (---*----)
    12      3     4.263      0.240   (---*----)
    13      3     4.897      0.680     (----*---)
    14      4     5.172      0.700       (---*---)
                                      --------+---------+---------+--------
POOLED STDEV =    0.962                   5.0       7.5      10.0

Fisher's pairwise comparisons

    Family error rate = 0.736
Individual error rate = 0.0500

Critical value = 2.035

Intervals for (column level mean) - (row level mean)

               1         2         3         4         5         6         7

     2   -2.7705
          0.4249

     3   -1.1783   -0.0055
          2.0171    3.1899

     4   -3.3095   -2.1367   -3.7289
         -0.4515    0.7213   -0.8709

     5   -0.4338    0.7390   -0.8532    1.6286
          2.5553    3.7280    2.1358    4.2539

1
     6   -1.2460   -0.0732   -1.6654    0.8032   -2.2035
          1.9494    3.1222    1.5300    3.6612    0.7855

     7   -5.8723   -4.6995   -6.2917   -3.8231   -6.8298   -6.2240
         -2.6769   -1.5041   -3.0963   -0.9651   -3.8408   -3.0286

     8   -6.5480   -5.3752   -6.9674   -4.4989   -7.5056   -6.8997   -2.2734
         -3.3526   -2.1798   -3.7720   -1.6408   -4.5165   -3.7043    0.9220

     9   -5.6894   -4.5166   -6.1088   -3.6402   -6.6469   -6.0411   -1.4148
         -2.4940   -1.3212   -2.9134   -0.7822   -3.6579   -2.8457    1.7806

    10   -1.5043   -0.3315   -1.9237    0.5581   -2.4541   -1.8560    2.7703
```

	1.4848	2.6576	1.0653	3.1834	0.3132	1.1330	5.7593
11	-2.5628	-1.3900	-2.9822	-0.5137	-3.5204	-2.9145	1.7117
	0.6326	1.8054	0.2132	2.3444	-0.5313	0.2809	4.9072
12	-0.3111	0.8617	-0.7305	1.7381	-1.2686	-0.6628	3.9635
	2.8843	4.0571	2.4649	4.5962	1.7204	2.5326	7.1589
13	-0.9449	0.2279	-1.3643	1.1043	-1.9024	-1.2966	3.3297
	2.2505	3.4233	1.8311	3.9623	1.0866	1.8988	6.5251
14	-1.1165	0.0563	-1.5359	0.9458	-2.0664	-1.4682	3.1581
	1.8725	3.0453	1.4531	3.5711	0.7009	1.5208	6.1471

	8	9	10	11	12	13
9	-0.7391					
	2.4563					
10	3.4461	2.5874				
	6.4351	5.5765				
11	2.3875	1.5289	-2.4499			
	5.5829	4.7243	0.5391			
12	4.6393	3.7806	-0.1981	0.6541		
	7.8347	6.9760	2.7909	3.8495		
13	4.0054	3.1468	-0.8320	0.0202	-2.2315	
	7.2008	6.3422	2.1571	3.2156	0.9639	
14	3.8338	2.9752	-0.9959	-0.1514	-2.4032	-1.7693
	6.8228	5.9642	1.7714	2.8376	0.5859	1.2197